OS5p+ User's Guide

(Preliminary)

Advanced Pulse Modulated Portable Chlorophyll Fluorometer

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OS5p+ 041714

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Chapter 1 • Introduction

Overview

This chapter provides you with a list of the equipment you should have received in your OS5p + shipment, basic software layout, an in depth discussion of all aspects of chlorophyll fluorescence measurement, and factors that produce reliable measurement. Chapter 2 talks about the OS5p+ hardware, and how things go together. Chapter 3 provides information on how to uses the OS5p+ to make measurements using the various chlorophyll fluorescence measuring protocols. Data management operation is covered in chapter 4. Examples of data files are presented in the appendix section along with maintenance information, technical specifications, and warnings.

Congratulations on your purchase of the OS5p+ Modulated Fluorometer. Please check the carton for any visible external damage. If you notice any damage, notify the freight carrier immediately. Follow their procedures for reporting and filing a claim. The carton and all packing materials should be retained for inspection by the carrier or insurer. The instrument may be used in the **Single User mode, or the Multi User mode**. The Multi User mode is designed for more than one user and designed to protect Data files and Preset measuring parameter files on Data Cards. In the Multi User mode, each individual will keep their own SD data card secure from others. When the card is entered into the instrument, Preset parameter test files and valuable Data files may be loaded into the active instrument memory. Data will be stored *only* on the memory card. The mode may be set in the main “Setup” screen.

List of Equipment

Carefully unpack the carton. You should have received the following items:

- 1 OS5p+ Fluorometer
- 1 PAR Clip
- 1 Universal Voltage battery charger
- 1 Trifurcated fiber optic light guide with built-in trigger switch
- 10 dark-adaptation cuvettes
- 1 Open body cuvette
- 1 OS5p+ owner’s manual (this document) on a USB drive
- 1 GB MMC/SD data card
- 1 USB cable
- 1 USB SD card reader
- 1 Nylon field bag
- 1 Shipping an storage case

If any item is missing, please contact your authorized Opti-Sciences agent.

Getting Started

Throughout this manual, you will be shown setup options and response messages. When an example of a program screen is given within a box, you may assume that this is shown, as it will appear on the OS5p+.

The user interface consists of a high-resolution color graphic touch screen LCD.

For editing parameters and making measurements, menu options are presented as icons or text legends. Measuring tests and parameter adjustments are all menu driven. For example, the curved arrow on the top left corner of most screens will bring you back to the previous screen.

The OS5p+ has default settings that allow the unit to work for many applications, however, changing the settings are very easily done.

Data is stored in an on-board system memory. This is based on flash memory so **no data will be lost if the main battery is depleted.** Data is stored on **data files** and measuring parameters are stored separately in **preset files**. Stored data may be transferred to other systems through use of the MMC/SD data card, or USB port. The data is output in comma delimited and carriage return separated ASCII strings, easily importable to most spreadsheet programs such as Excel, Mat Lab, or other comma delineated spread sheets. There is a **multi – user** mode that may be selected from the diagnostic screen. When selected, data files and preset files, or parameter settings, are only stored in separate files on data cards, not the built in memory.

Tests Available

The OS5p+ is equipped to make several different kinds of tests including: Dark adapted tests that include: F_V/F_M and F_V/F_O, Strasser protocol OJIP with all of its parameters, and Vredenberg OJIP quenching protocol with values reported in $F_{(t)}/F_O$, Quenching protocols include Kramer lake model, Hendrickson lake model with NPQ, puddle model, and quenching relaxation protocol for q_E, q_M, q_Z, q_T, and q_I. and Light adapted tests include Quantum yield of PSII or $\Delta F/Fm'$ or Y(II), a multiple phased single saturation flash for F_M' correction after Loriaux (2006) & (2013), Rapid Light curves and Light curves. Quenching is both dark adapted and light adapted.

A PAR Clip is included with the OS5p+ for ETR, PAR, and Leaf temperature measurement. PAR Clips are highly recommended for field and laboratory measurement of Y(II) and ETR. Because Y(II) value vary not only with plant stress, but also with light level, only samples at very similar light levels and vary similar light histories should be compared. Sun leaves will respond differently than shade leaves to different light levels. Review the “Desk Top Plant Stress Guide” available for free from Opti-Sciences Inc. at www.optisci.com for the type of test that is best suited to your research.

The quenching option details:

1. Dave Kramer’s lake model parameters (Kramer 2004) - Y(II), q_L, Y(NPQ), and Y(NO)

2. Luke Hendrickson’s lake model parameters with Klughammer’s resurrection of NPQ from the puddle model are included in this protocol - Y(II), Y(NPQ),Y(NO), and NPQ.

3. Puddle model quenching parameters q_N, q_P, and NPQ.

4. A relaxation protocol for subdividing NPQ into q_E, q_M, q_Z, q_T, and q_I. This protocol may be used with NPQ in the puddle model, the Kramer lake model, or the Hendrickson lake model.

The OS5p+ also includes a Strasser OJIP plant stress measuring protocol, and a Vredenberg OJIP quenching measurement protocol. In the Strasser OJIP protocol, all of the Strasser measuring parameters are available in the data file, while the most popular parameters are provided with direct readout on the measuring screen including PI_{ABS}. The Vredenberg protocol allows a high time resolution method for the investigation of non-photochemical quenching. It is a scripting based protocol that allows the actinic light to be turned on or off where desired, even after several microseconds.

F_V/F_M and Y(II) Definitions:

Actinic light source – This is any light source that drives photosynthesis. It may be the Sun, or an artificial light. Some higher end fluorometers contain one or more built-in artificial actinic light sources for experimentation with specific repeatable radiation (or light) levels. The OS5p+ uses a stable high intensity white light LED that is designed for pre-illumination of samples and quenching measurements at stable intensities when used with the PAR Clip. The LED provides an intense blue spectrum that ensures proper chloroplast migration as found in nature. This light source also allows the measurement of qM or chloroplast migration (Cazzaniga 2013).

Dark-adapted or Dark Adaptation – This is a term that means that an area of a plant, or the entire plant, to be measured has been in the dark for an extended period of time before measurement. Dark adaption requirements may vary for dark-adapted tests. Dark adaption times of twenty minutes to sixty minutes are common, and some researchers use only pre-dawn values. Dark-adapted measurements include F_V/F_M, and non-photochemical quenching parameters. Longer dark adaption times are common for quenching measurements. In this case, it is common to use times of twelve hours, or overnight. For a detailed discussion of dark adaptation, refer to the application note on dark adaptation.

Far red light – is a light source that provides light above 700 nm to drive PSI, drain PSII of electrons, and allow the rapid re-oxidation of PSII. It is used extensively for the determination of quenching parameters in Quenching protocols, and for pre-illumination and rapid re-oxidation of PSII in F_V/F_M measurements. F_o' is used in determining Kramer's quenching parameters, as well as puddle model, q_N. They require the use of far red light to determine quenched F_o.

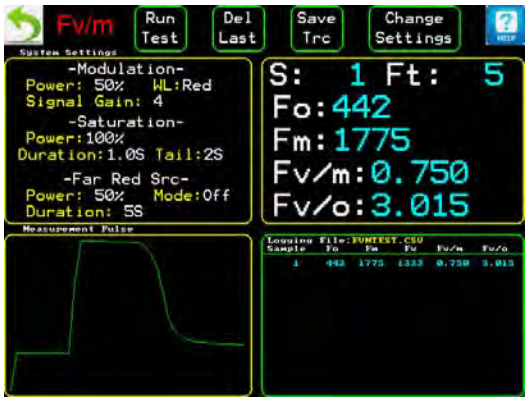
F_M - is maximal fluorescence measured during the first saturation pulse after dark adaption. Fm represents multiple turnovers of Q_A with all available reaction centers closed. All available energy is channeled to fluorescence.

F_S - also known as F' at steady state, is the fluorescence level created by the actinic light . Initially the value is high and then decreases over time to steady state values due to the initiation of electron transport, carboxylation, and nonphotochemical quenching. F_s has also been used to designate steady state F' conditions.

F_M' – is the saturation flash value that is not dark-adapted. They are at a lowered values due to NPQ or non-photochemical quenching. When this parameter has reached steady state, it is used to calculate Yield of PSII or Y(II) or ΔF/Fm' along with F_s. $F_{M'}$ at steady state is also used to calculate q_N, NPQ, q_P, qL, Y(NPQ), Y(NO), q_E, q_T, and q_I.

F_O is minimal fluorescence after dark adaptation. It is measured with a modulated light intensity too dim to drive chemical reduction of Q_A and yet bright enough to detect "pre-photosynthetic" antennae fluorescence.

F_t – is the current instantaneous fluorescent signal shown on the fluorometer measuring screen. It is used to set the modulated light source intensity. See setting the modulated light source intensity.



F_V/F_M measuring Screen

Fast -Dark Adapted Fv/Fm
Usually less than 2 to 4 seconds but may take longer with pre-illumination with far red light

F_V/F_M = $(F_M - F_O) / F_M$ Maximum quantum yield. This is a dark adapted test. This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers (Kitajima and Butler, 1975). Another way to look at F_V/F_M is a measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centers were open or oxidized. 0.79 to 0.83 is the approximate optimal value range for most land plant species with lowered values indicating plant stress. It is important to properly dark adapt samples for this test. F_O will be raise and F_M will be lowered if dark adaption is inadequate. Since dark adaption requirement can vary with species and light history, and type of dark adapted test, testing should be done to ensure proper dark adaption, (See the section on dark adaptation). This test is a normalized ratio.

F_V/F_O = $(F_M - F_O) / F_O$ This is a more sensitive plant stress detector than F_V/F_M. It is important to properly dark adapt samples for this test. F_O will be raise and F_M will be lowered if dark adaption is inadequate. Since dark adaption requirement can vary with species and light history, and type of dark adapted test, testing should be done to ensure proper dark adaption, (See the section on dark adaptation). This test is a normalized ratio. Measurements commonly range from 0 to 4.

Modulated light source -This is the light source that makes light adapted quantum photosynthetic yield measurements possible along with direct measurements of F_o and F_o'. The modulated light source is used at an intensity range that is too low to drive photo-chemical

reduction of Q_A and yet allows fluorescence measurement of pre-photosynthetic F_o, and photosynthetic F_o'. This light source is turned off and on at a particular frequency. The frequency is adjusted automatically for optimal application usage. Intensities are adjusted between 0 to 0.4 µmols. The intensity and frequency must be set differently for light and dark adapted methods, Vredenberg OJIP quenching and quenching relaxation tests. It is critical to adjust the intensity of this lamp correctly in dark adapted protocols to prevent driving photo-chemical reduction of Q_A in F_V/F_M, and quenching measurements. For more details see the application note on dark adaptation. The OS5p+ has an automated routine that can be used to automatically set the modulated light intensity correctly. It can also be set manually if desired. The frequency is set automatically for the test that is being used.

PAR – Photosynthetically Active Radiation between 400nm and 700nm. Measured in either µmls or µE. The term PAR means photosynthetically active radiation in the wave band between 400-700 nm. PAR can be measured in different dimensions such as Watts per meter or in micro-Einsteins or micro-moles. When using a PAR Clip, dimensions will always be in micro-moles.

PAR Clip – This is a fluorometer accessory that allows the measurement of PAR or PPFD and Leaf Temperature along with Yield measurements. Since Y(II) change with PAR radiation (or light) levels and temperature levels, the ability to record Yield values with these parameters provide control over important variables. Since changes in Y(II) can be due to changes in plant stress or light level, it is important to control and measure light level when comparing values. A PAR Clip allows the calculation of relative ETR or Electron Transport Rate. When used with internal fluorometer actinic illuminators, it will measure reproducible and repeatable controlled values. PAR clips are recommended for field use with quantum photosynthetic yield measurements. See the section on quantum photosynthetic yield for an in depth discussion.

PPFD - Photosynthetic Photon Flux Density is the photon flux density of PAR. Measured in either µmls or µE., PPFD, or "photosynthetic photon flux density", is the number of PAR photons incident on a surface in time and area dimensions (per meter squared per second). These terms are equivalent for PAR Clip leaf radiation measurements. Furthermore, both can be presented in either of the equivalent dimensions, micro-moles (µmls) or micro-Einsteins (μ_E).

Y(II) or Yield Measuring Screen

Fast -Light Adapted Y(II)
Yield of PSII Test –
Usually less than 2 to 4 seconds.

Quantum Yield of PSII = Y(II) or $(F_M' - F_S) / F_M'$ -This test is also known as $\Delta F/F_M'$. Yield of PSII is a fast light adapted test taken at steady state photosynthesis levels. It provides a measure of actual or effective quantum yield. This ratio is an estimate of the effective portion of absorbed quanta used in PSII reaction centers. (Genty, 1989) It is affected by closure of reaction centers and heat dissipation caused by non-photochemical quenching. It allows investigation of the photosynthetic process while it is happening. No dark adaption is required. When a sample is exposed to light, it normally takes several minutes for photosynthesis to reach steady state. According to Maxwell and Johnson (2000) it takes between fifteen to twenty minutes for a plant to reach steady state photosynthesis. To obtain a reliable yield measurement, photosynthesis must reach steady state. Recently is was found that under high actinic light conditions it can take 20 minutes to 35 minutes to reach steady state photosynthesis due to chloroplast migrations (Cazziniga 2013).

This is usually not a concern when using ambient sunlight or artificial greenhouse light, however, clouds and light flecks below a canopy level can cause problems. If one uses a built in fluorometer actinic illuminator to measure yield, make sure that steady state photosynthesis has been reached (See the discussion on Quantum Yield of PSII for more information). Remember that Ambient Sun light contains FAR red illumination for activation of PSI. It is something to consider when using an internal illuminator for Y(II) measurements. Far Red illumination is an option when using internal actinic illumination for yield measurements. See the section regarding an in depth discussion on quantum yield of PSII.

Y(II) has been found to be more sensitive to more types of plant stress than F_V/F_M, however one must only compare measurements at the same light level, as the value changes at different light levels. A PAR clip should be used with the fluorometer to measure Y(II) in all field applications. This allows for proper comparisons of values and the determination of ETR or electron transport rate, a parameter that includes both yield and actinic light level. See the Stress guide for more details.

Light adapted measurements include Y(II) or $\Delta F/F_M'$, ETR, PAR (or PPFD), and Leaf Temp, quenching measurements, Rapid Light Curves, and Light Curves.

Saturation pulse - is a short pulse of intense light designed to fully reduce all available PSII reaction centers. For higher plants, the optimal duration of the saturation pulse is between 0.5 seconds and 1.5 seconds (Rosenqvist and van Kooten 2006). It is typically a white light that has to be high enough to close all available PSII reaction centers. With leaves that have a very intense actinic light history, this may not be possible using a standard square flash. For these samples, Multi-Flash is recommended. This is a multiple phased single saturation flash developed by Loriaux (2006 & 2013) It is designed to correct for this issue. See the section on Multi-Flash for a detailed explanation. On the OS5p+ an LED light source is used. Opti-Sciences uses 0.8 seconds as the default saturation pulse duration for higher plants. And 1.1 seconds in the Multi-Flash F_M' correction protocol after Loriaux 2013. The duration is adjustable from 0.1 to 2.0 seconds; however, the OS5p+ uses an eight point 25 ms rolling average to determine F_M and F_M'. This ensures that the optimal measurement F_M, or F_M' values even if the duration is set too long as long as the duration is long enough.

μ_E – is a **micro Einstein.** This a dimension that involves both time and area. It is equivalent to the **µmol**. Both terms have been used extensively in biology and radiation measurements.

µmls - is a **micro mol** (also abbreviated µmol, or µmol $m^{-2}s^{-1}$) . This a dimension that involves both time and area (per meter squared per second) . It is equivalent to the micro Einstein. Both terms have been used extensively in biology and radiation measurements.

µmol - s a **micro mol** (also abbreviated µml, or µmol $m^{-2}s^{-1}$) . This a dimension that involves both time and area (per meter squared per second) . It is equivalent to the micro Einstein. Both terms have been used extensively in biology and radiation measurements.

1µE = $1\ \mu mol\ m^{-2}\ s^{-1} = 6.022 \times 10^{17}$ photons $m^{-2}\ s^{-1}$

For Quenching definitions and equations go to the Quenching section

Variable Chlorophyll Fluorescence – Overview (2014)

Chlorophyll "a" absorbs light most effectively in the red and blue parts of the visible spectrum. Chlorophyll fluorescence is light that is re-emitted at a longer wavelength after being absorbed by chlorophyll molecules at shorter wavelengths. Variable chlorophyll fluorescence is only observed in chlorophyll "a" in photosystem II. By measuring the intensity and nature of variable chlorophyll fluorescence, and using protocols that have been developed, plant physiology can be investigated (Baker 2004). The variable nature of chlorophyll fluorescence allows research into the light reaction of plants, plant photo-protection mechanisms, heat dissipation, correlation with photosynthesis carbon assimilation, and measurement of most types of plant stress at usable levels (Baker 2004). As stated earlier, the sole origin of variable chlorophyll fluorescence is chlorophyll "a" in photosystem II (Zhu 2005). Light energy entering photosystem II can be converted to chemical energy by photochemistry. It can also be re-emitted as chlorophyll fluorescence or it can be re-emitted as heat. These three processes are in competition, so that when photochemistry output is high, chlorophyll fluorescence and heat are lower. Conversely, if fluorescence is maximized, then the other two paths are minimized.

While photosystem I does emit chlorophyll fluorescence as well, it is at a much lower level and it is not variable. For that reason, chlorophyll fluorescence of photosystem II is of much greater interest. (Schreiber 2004)

Photosynthesis is comprised of a light reaction and a dark reaction. The light reaction converts light energy into chemical energy that can be used in the dark reaction. The dark reaction uses the energy molecules NADPH and ATP, created by the light reaction, to produce simple sugars in conjunction with the assimilation of CO_2 from the air. The relationship between the two is related, and may be correlated, but it is not always straightforward (Rosequist and van Kooten). Correlation of variable chlorophyll fluorescence in the light reaction, and carbon assimilation in the dark reaction has a linear relationship in C_4 plants, and a curve-linear relationship in C_3 plants (Schrieber 2004). This correlation in C_3 plants can break down under special conditions that create photorespiration such as drought and heat stress. Under photorespiratory conditions,

oxygen and possibly other electron sinks, alternatively use the energy molecules generated in the light reaction, even after leaf carbon assimilation has been reduced. Under these conditions, chlorophyll fluorescence levels may be unchanged until severe plant stress occurs (Baker 2008), (Flexas 1999). In C_4 plants, there is no significant photorespiration. Fortunately, special chlorophyll fluorescence assays or methods have been developed to overcome the most interesting problem areas including drought stress in C_3 plants, nitrogen stress in C_3 plants and C_4 plants, and heat stress in C_3 and C_4 plants (Desk Top Plant Stress Guide).

How does the variable chlorophyll fluorescence work?

(Dark adapted tests)

Upon illumination of a leaf that is dark-adapted overnight, or dark adapted by artificial means, there is a rapid rise in fluorescence from Photosystem II (PSII), followed by a slow decline. This displays the variable nature of chlorophyll fluorescence in photosystem II. It was first detailed by Kautsky in 1931, and this is called the Kautsky Effect. (Govindgee 2004) (Kautsky 1931) In Photosystem II, light is absorbed by accessory pigment-protein complex molecules called antenna (Zhu 2005), and transferred to PSII reaction centers. Zhu divides antenna into peripheral antenna and core antenna. Core antenna are tightly bound chemically and adjacent to the reaction center. Peripheral antenna are near the core and chemically bound strongly, at a medium level, or loosely. They are; however, in close proximity to either photosystem II or photosystem I reaction centers (Schneider 2013).

In the photosystem II reaction center are D_1 and D_2 pigment-protein complexes that coordinate the specialized chlorophyll “a” photoactive reaction center structure, P_{680}. (Papageorgiou 2004). There are different models that show P_{680} acting as various types of dimer (Papageorgiou 2004), (van Gronelle 2004), (Razewski 2008), or a monomer (Takehashi 2009); however, the Zhu (2005, 2012) papers, written by some of the most prominent chlorophyll fluorescence researchers, focus on fluorescence and provide a conservative approach that is currently the most accepted. The core antenna complexes are known as CP43 and CP47. They are chlorophyll-protein complexes that are adjacent and associated with the D_1D_2 P_{680} PSII reaction center. (For more details concerning these structures see Diagrams C or refer to the application note on Variable chlorophyll fluorescence at www.optisci.com .

There are several different pigment types associated with peripheral antenna including: Chlorophyll a, Chlorophyll b, lutein, xanthophylls, beta carotene, and lycopene. The antenna absorb light in different wavelength ranges, and transfer the energy to nearby photosystem I and II reaction centers. As the energy transfer occurs to the reaction center, a small amount of energy is lost to passive heat loss. The antennas transfer energy to photosystem II reaction centers and to photosystem I reaction centers. Both photosystems are located in thylakoid membranes. Thylakoids are lumened structures stacked, inside plant chloroplasts. They are pictured in the drill down diagrams below (Diagram B). There are two basic types of photosystems called either photosystem II, or photosystem I. (Diagram C). While it has been shown that chlorophyll “b” can show a slight fluorescence when energy can not be transferred to chlorophyll “a”, the emission spectrum in the 660 nm to 665nm range, is normally filtered out by chlorophyll fluorometers and it does not directly affect F_O, or F_V/F_M (Govingee 1978). There has been no fluorescence observed in chlorophyll “b” when energy transfer to chlorophyll “a” is normal. (Govingee 1978).

There are also two varieties of photosystem II reaction centers that affect variable chlorophyll fluorescence. Energy is transferred to either Q_B^- reducing reaction centers, that are capable of being used in photochemistry, or to Q_B^- non-reducing reaction centers, that are not capable of photochemistry. Q_B^- non-reducing reaction centers do not transfer their energy to other reaction centers, and the absorbed energy is reemitted as either heat or non-variable chlorophyll fluorescence at a low level (Diagram C). Q_B^- non-reducing reaction centers have smaller core antenna, an oxygen evolving complex, and no peripheral antenna. There is also no electron transfer beyond Quinone A or Q_A. (Quinone B is designated Q_B). A higher number of Q_B^- non-reducing reaction centers in the leaf therefore increase the minimum fluorescence, F_O, which is measured in a dark adapted state, and decreases the F_V/F_M measurement parameter to be discussed in detail later (Zhu 2005).

Q_B^- reducing reaction centers that can be used in photochemistry, can be either opened or closed. They are open if they are chemically oxidized, and they are closed if they have been chemically reduced. Closed Q_B^- reducing reaction centers can transfer additional energy to other open Q_B^- reducing reaction centers. In a properly dark adapted state, most or all Q_B^- reducing reaction centers will be open (Zhu 2005).

When a, Photosystem II, Q_B^- reducing reaction centers receive an adequate threshold of light energy, it drives something called charge separation that occurs in photosystem II (Zhu 2005).

This, and the electron transfer to the A_O molecule in photosystem I are the only steps where light energy is converted into chemical energy (Zhu 2005).

At charge separation in photosystem II, an electron is transferred from P_{680}, the primary electron donor, to the primary electron acceptor, pheophytin. The chemical process for charge separation is shown in diagram A. During this process, an electron is added from Tyrozine, Y_Z, generated from the Oxygen Evolving Complex, through the Mehler reaction. The oxygen evolving complex involved in this process is also shown in the diagram C below (Zhu 2005).

The energy levels of the remaining steps in the light reaction of photosystem II are all down hill in oxidation-reduction reactions (Zhu 2005).

Measuring fluorescence in a dark adapted state, starts by measuring minimum fluorescence generated by peripheral and core antenna before any Q_A has been chemically reduced (Zhu 2005). This is commonly done using a modulated chlorophyll fluorometer that excites minimum fluorescence. The modulated light source is adjusted high enough to allow minimum antenna fluorescence measurement, but it is set low enough to prevent the reduction of any Q_A. Additional sources of minimum fluorescence also include Photosystem I, and PSII Q_B^- non-reducing reaction centers (Zhu 2005), (Opti-Sciences F_V/F_M checklist application note). The rise of variable chlorophyll fluorescence starts at charge separation and continues through the reduction of Q_A, Q_B, and the reduction of the Plastoquinone Pool. Evidence shows that the rise ends with the re-oxidation of PQH_2 (Plastoquinol) to PQ (plastoquinone) by the Cytochrome b_6f complex. Maximum variable fluorescence or F_M, occurs when $Q_AQ_B^{2-}$ and PQH_2 are at a maximum (Zhu 2005). The height of F_M is affected by the size of the Plastoquinone pool and the rate constant for reoxidation of PQH_2 to PQ. A higher k_{ox}, reoxidation constant, or a larger plastoquinone pool reduces the F_M value (Zhu 2005).

Different types of plant stress affect PSII differently, therefore one should consult the “Desk Top Plant Stress Guide” on this disc to determine the best measuring protocol or special assay before working. Research referenced in the Plant Stress guide shows that while some types of plant stress affect chlorophyll fluorescence of a plant in a dark adapted state (F_V/F_M), measuring some types of plant stress at a sensitive level requires the light adapted Y(II) or $\Delta F/F_M$’ or special assays.

(Diagram A) This diagram is adapted from Zhu (2005) page 116, with more in-depth descriptions for purposes of understanding. It represents one reducing Q_B^- PSII unit.

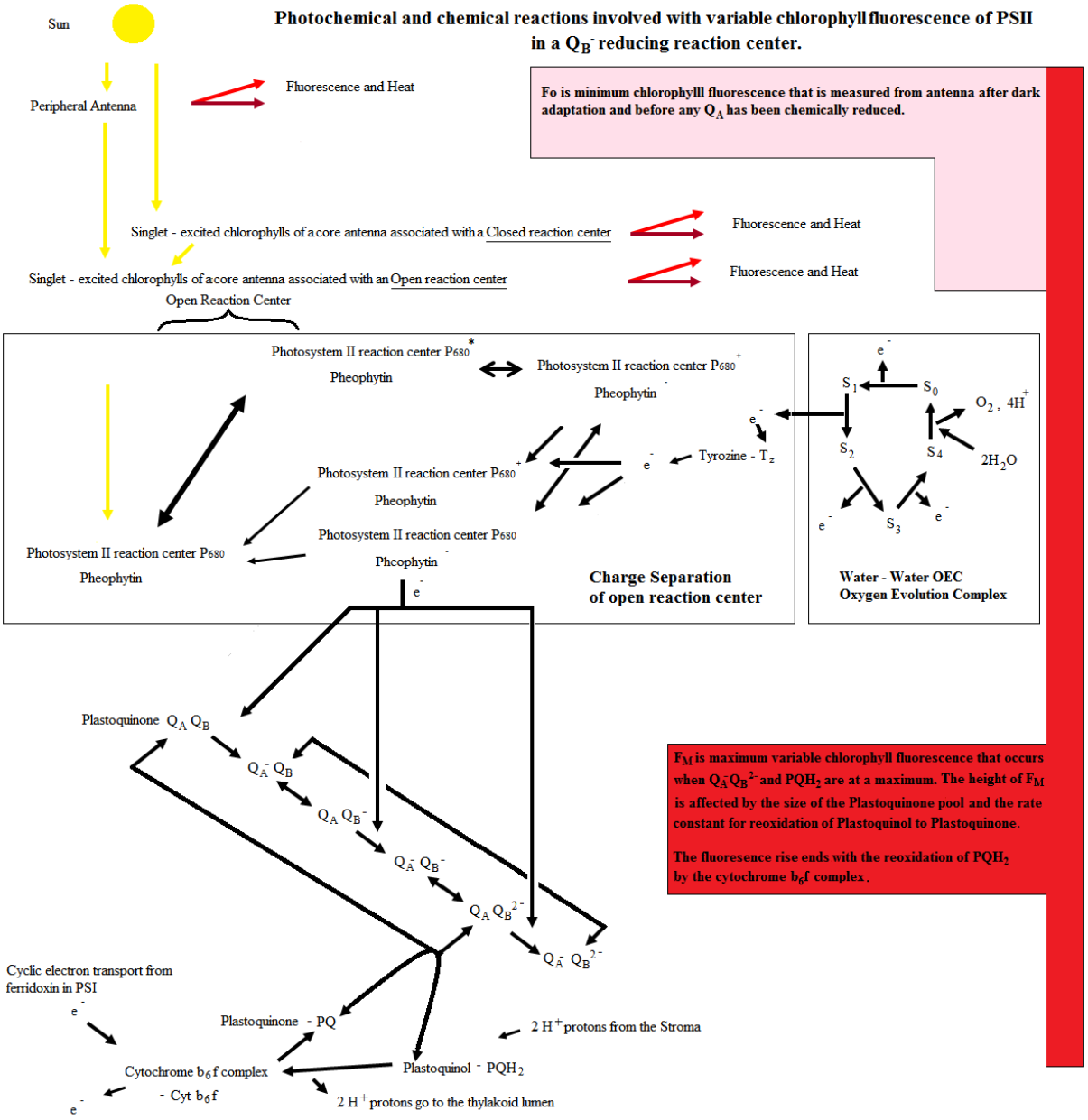


Diagram B

Drill down diagram of variable and non-variable chlorophyll fluorescence

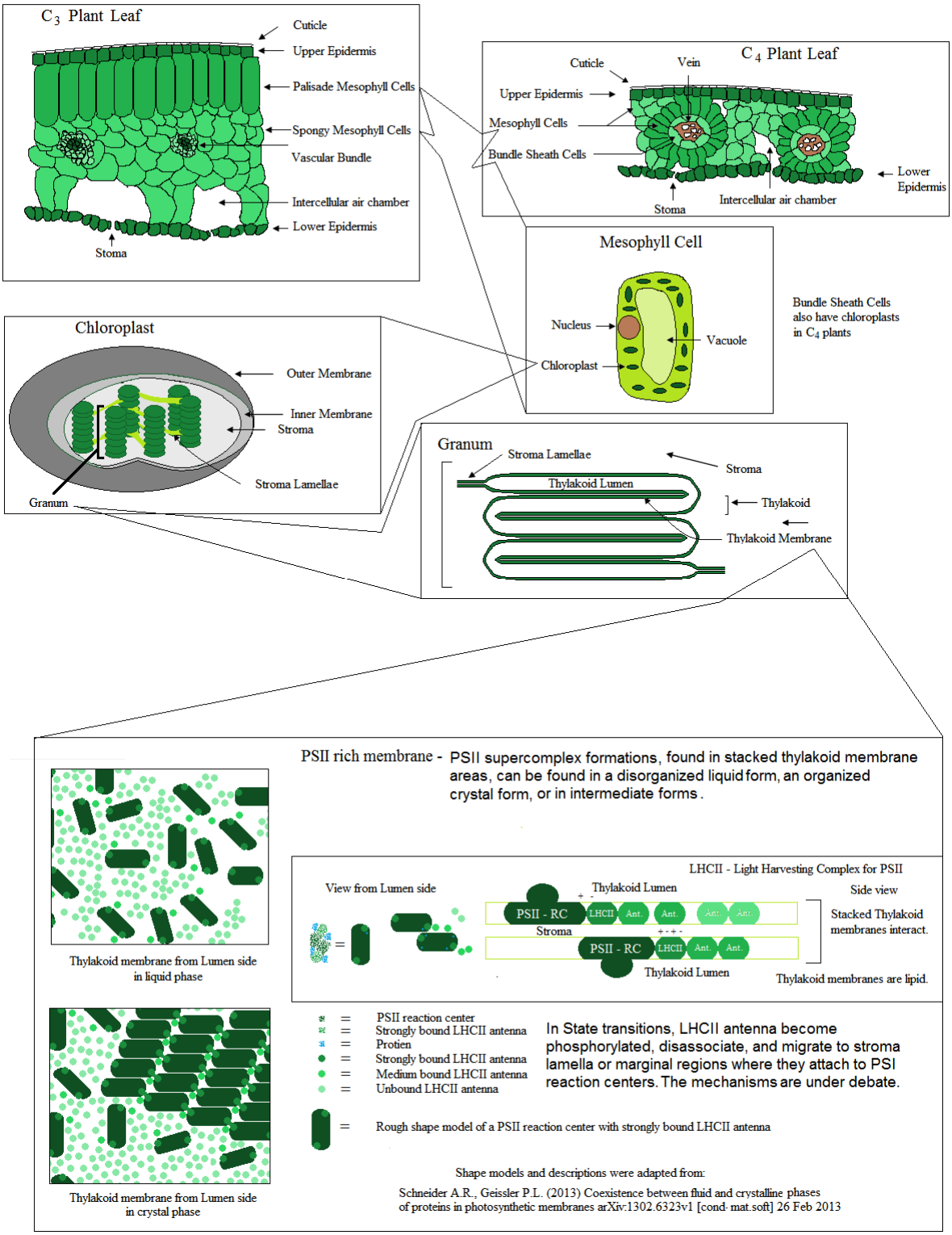
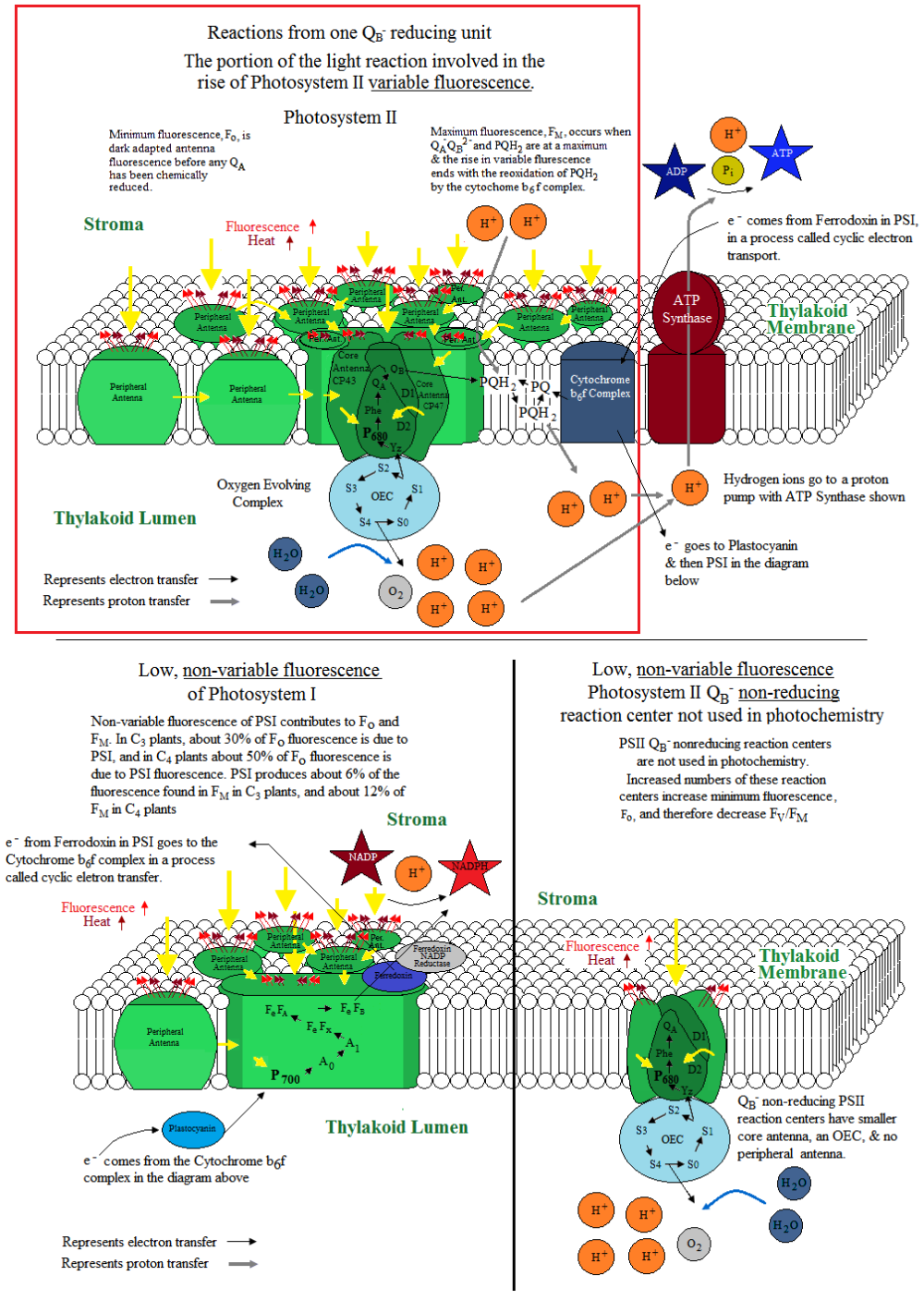


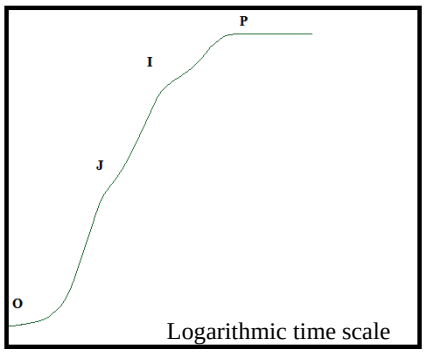
Diagram C

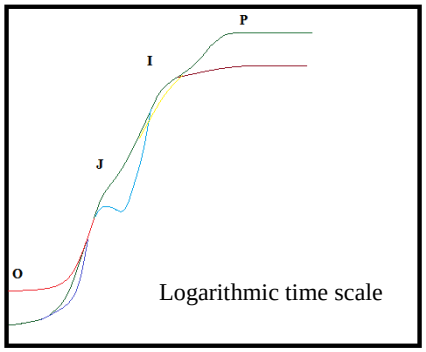
(Diagram C) This is a drill down diagram of the mechanisms that affect variable chlorophyll fluorescence. It designed as a more visual representation of the most accepted understanding of the sources of chlorophyll fluorescence and variable chlorophyll fluorescence. Chlorophyll fluorescence generating sources for Photosystem II were taken from Zhu 2005, & 2012. Photosystem I information was taken from Schreiber (2004).



OJIP and F_V/F_M - Understanding the fluorescence rise and steps.

If the rise in chlorophyll fluorescence is examined with high speed time resolution in the range of microseconds, and milliseconds, specific steps appear during the rise. The following descriptions represent a synopsis of information available from a paper written by Xin-Guang Zhu, Govindjee, Neil R. Baker, Eric deSturler Donald R. Ort, and Stephen P. Long in 2005. The information was reaffirmed in a second paper in 2012 by Xin-Guang Zhu,Yu Wang, Donald R. Ort, and Stephen P. Long. These are some of most respected names in chlorophyll fluorescence and photosynthesis. While there is still some debate regarding some of the details of the OJIP fluorescence rise, this approach is the one that is most accepted.



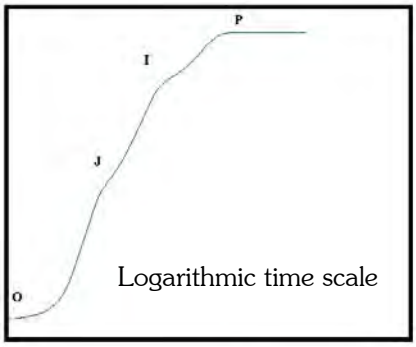


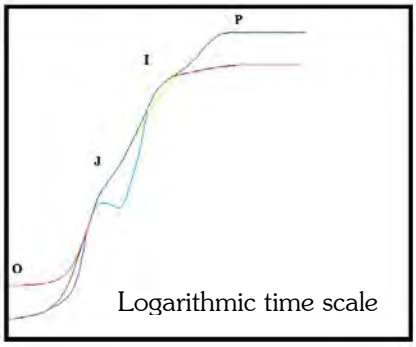
O – is commonly measured at 20 µsecs. after the start of actinic illumination in continuous fluorometers, and is not equal to F_O measured by modulated fluorometers. Continuous fluorometers use linear regression analysis to estimate F_O, or minimum fluorescence in a dark adapted state, before any Q_A has been chemically reduced.

The height of both O and F_O are affected by the ratio of the size of peripheral antenna to core antenna. A larger ratio of peripheral antenna causes O and F_O to be lower.

Both O and F_O are affected by the ratio of Q_B^- non-reducing reaction centers to Q_B^- reducing reaction centers. As the ratio of Q_B^- non-reducing reaction centers increases, both O and F_O values increase. This is shown in the bottom diagram. The green line represents a sample with significantly fewer Q_B^- non-reducing reaction centers, and the red line represents a much higher percentage of Q_B^- non-reducing reaction centers.

O to J – The slope of the rise in fluorescence is affected by the probability that excitation energy will migrate from a closed Q_B^- reducing reaction center to an open Q_B^- reducing reaction center. Higher probability delays the rise as shown in the lower diagram in dark blue. Q_B^- non-reducing reaction centers do not transfer energy to open Q_B^- reducing reaction centers. Energy absorbed by these reaction centers is converted to heat and fluorescence only. They are not involved in photochemistry. The O-J rise represents the photochemical reduction of pheophytin and Q_A. J represents maximum values for $Q_AQ_B^-$ and $Q_A^-Q_B^-$. J becomes more defined and lower if the dark adapted Oxygen Evolving Complex ratio of the specific states of S_1 to S_0 move from 1:0 to 0:1. The Dip after J becomes more defined with a higher S_0 value. It provides a greater P_{680}^+ concentration that is a strong fluorescence quencher. This dip is shown in the lower diagram in light blue. A separate new step called the K step can appear at 300 µsecs. It only appears at high light levels (Vredenberg 2004), when there is severe nitrogen, iron, or sulfur deficiency (Strasser 2004). The Zhu 2005 paper show that the timing of J is somewhat variable. However, in the Strasser JIP protocol, used for plant stress measurement, it is fixed at 2 msec.





J to I - This rise represents the photochemical reduction of Q_B . "I" represents the first shoulder in the $Q_AQ_B^{2-}$ chemical equation that ends at P with a maximum for $Q_A^- Q_B^{2-}$. If properly dark adapted, the J to I rise starts with the ratio of $Q_B : Q_B^- = 1:0$ and ends with the ratio at 0:1.The dark adapted ratio prior to light exposure of $Q_B : Q_B^-$ affects the slope and height of "I" as shown in the lower diagram by the yellow line.

Again, the Zhu group shows that the time to reach I is also somewhat variable. However, in the Strasser JIP protocol, used for plant stress measurement, it is fixed at 30 msec.

P = F_M or Maximum variable chlorophyll fluorescence. This value represents a maximum for chemical values of $Q_A^-Q_B^{2-}$, & PQH_2. The rise in fluorescence ends with the cytochrome b_6f complex re-oxidizing PQH_2 to PQ. The height and slope of the rise to P or F_M are affected by the reoxidation rate constant of PQ, k_{ox} , and by the size of the plastoquinone pool. A higher rate constant and a larger PQ pool reduce the value for P. The time to reach P is variable in the Zhu paper and in the Strasser JIP protocol. A high rate constant reduces the time to reach P, and a larger PQ pool extends the time to reach P. The time to reach P is reported in the Strasser protocol. A larger PQ pool is shown in the lower diagram in dark red crimson.

In 2004, Wim Vredenberg discovered that the OJIP graph changes dramatically at different actinic light levels. In fact, the K step only appears under very high light levels, and under specific severe plant stress conditions. For this reason, it is common to calibrate the light source of OJIP instruments, to ensure comparable results. The $OS30p_+$ provides automatic actinic light calibration when the instrument is turned on. (Vredenberg 2004)

The quality of light can also be a factor. It has been found that red actinic light penetrates the entire leaf, while blue light does not. For this reason, it has been common to compare work done using the same type of saturating actinic light sources. The OS30p+ offers a red calibrated light source with intensities that may be set at 3,500 µmols for the Strasser protocol and up to 6,000 µmols for other work. Various light sources are used for measurement of F_V/F_M. Industry options include, red, red and blue, white light halogen, white LED, and Xenon light sources. A paper comparing xenon and red light saturating light sources provided results that correlated well with slightly lower values using a red light source (Cessna 2010). The paper found poorer correlation with blue saturating light. For longer measuring protocols used in quenching measurements, and light curves, or for extended pre-illumination of shorter light adapted tests, white actinic light sources have advantages. The apertures of plant stoma are mediated by blue actinic light (Kinoshita 2001). In addition, chloroplast migration can be responsible for up to 30% of nonphotochemical quenching at high actinic light levels at steady state photosynthesis. It only occurs when white actinic light is used or actinic light sources with an intense blue spectrum. There is no significant chloroplast migration with intense red light. Chloroplast migration also changes leaf absorptance (Cazzaniga 2013).

Photochemical and Non-photochemical quenching.

After proper dark adaptation to a known state, a leaf is exposed to a photosystem saturating light. Initially, a maximum amount of the saturating light, absorbed by the leaf, and used in F_V/F_M, goes to variable chlorophyll fluorescence with smaller amounts going to unregulated heat dissipation and photochemistry. The same thing is true when using a saturating actinic light in OJIP protocols for the initial rise of fluorescence.

There are other mechanisms, that are slower reacting, that affect variable chlorophyll fluorescence. After dark adaptation, and the initial rise in chlorophyll fluorescence, these mechanisms begin to respond. Depending on the type of plant, peak fluorescence is maintained from 0.5 seconds to 1.5 seconds in land plants (Schreiber 1995), and from 25 milliseconds, to 50 milliseconds in algae (Schreiber 1995). The fluorescence output then begins to drop due to the initiation of photosynthesis where more light is used in photochemistry, a process called photochemical quenching. This, and photo-protective mechanisms, start to adapt to existing actinic light levels. The xanthophyll cycle and the Δ Ph of the thylakoid lumen convert absorbed light into regulated heat dissipation, a form of non-photochemical quenching, considered to work as photoprotective mechanisms. At higher light levels, there is more non-photochemical quenching. This process takes about several seconds to minutes in greenhouse plants (Lichtenthaler 2004), but it can take up to seven minutes in field plants (Baker 2008). q_E is a parameter that is used to measure the non-photochemical quenching photoprotective mechanisms. This parameter is used in conjunction with quenching relaxation protocols (Muller 2001). q_L from the lake model and q_P from the puddle model are parameters designed to measure photochemical quenching or a measure of open PSII reaction centers.

There are still other slower acting mechanisms that continue to lower fluorescence output after the initial fluorescence rise. Some prominent researchers see evidence that these intermediate but slower component mechanisms that are part of an NPQ measurement, may not be the same in all photosynthetic organisms. There could be a relationship between the phosphorylation found in state transitions and NPQ regulation found in some monocots (corn, barley and rice). There is also some strong evidence that q_T fluorescence, from state transitions, exists in the green algae Chlamydomonas reinhardtii. (Depège N., Bellafiore S., Rochaix J-D., 2003).

The relevance of state transitions measured as q_T fluorescence is highly questionable for a number of land plants including Arabidopsis. The evidence shows that changes previously reported as q_T in quenching relaxation tests are not due to state transitions at higher light levels or saturating actinic light intensities. The latest evidence points to chloroplast migration and the resulting reduced leaf photon absorption as the source of fluorescence change during light adaptation and during quenching relaxation, in dicot land plants at the very least (Cazzaniga 2013).

What causes the non-photochemical quenching (NPQ) fluorescence relaxation change that is greater than a few minutes and less than thirty five minutes? In 2010 (Nikens) it was found that q_T did not exist at near saturation light conditions. It was though that it must be an unknown slower acting mechanism related to zeaxanthin, and it was named q_Z (Nilkens 2010). Since that time, there has been new evidence. Under higher light levels and near saturating light conditions, the latest data supports chloroplast migration as the source of fluorescence change and quenching relaxation. This research also affects the type of actinic light sources that should be used to

measure most light adapted parameters, quenching measurements, and under high light conditions, the times recommended for dark adaptation, and the time required to reach steady state photosynthesis under high light conditions. (Cazzaniga 2013).

Until recently, it was believed that as a plant goes from a dark adapted state to a high light level or from a high light adapted state to a dark adapted state, there were three basic mechanisms involved in chlorophyll fluorescence measurement of nonphotochemical quenching (NPQ) ; q_E, q_T, and q_I. q_E can be described as a rapid photo-protective adjustment of photosystem II caused by ΔpH of the thylakoid lumen and the xanthophyll cycle. It can take q_E several seconds to minutes to adjust, and it tends to be longer in field plants (Baker 2008, Murchie 2011, Nilkens 2010). Traditional q_T was thought to be caused by state transitions. q_T could take up to fifteen or twenty minutes has been described as a fluorescence change that overlapped somewhat with q_E. Changes that took longer were related to q_I or photoinhibition (Ruban 2009). There is now significant evidence to show that the fluorescence change measured as q_T in quenching relaxation measurements is likely due to other chloroplast migration at least under high actinic light conditions in many land plants.(Cazzaniga 2013).

State transitions – classical view:

According to classical state transition theory, state transitions are thought to be a low light level survival mechanism that allows balancing of light between photosystem II (PSII) and photosystem I (PSI). It was believed that LHCII antenna trimers, or peripheral phosphorylated light harvesting complex II antenna, migrated from PSII complexes, to PSI complexes. The movement would occur from one thylakoid membrane to another when they were very close or adjacent to one another. The movement and reaction took place on the stroma side of the thylakoid membrane allowing the LHCII antenna to serve as a PSI antenna. When dephosphorolated, the LHCII antenna favored movement back to PSII. The result was that at low light levels, movement was favored to PSI. LHCII phosphorylation was a prerequisite for dynamic regulation of relative balance of PSI/PSII excitation under artificially induced state transitions with different qualities of light. However, this process has not been viewed, without reservation, in PSII rich thylakoid membranes.

State transitions – a more recent view:

A more recent view of state transitions is as follows: There is no clear evidence to support the actual movement of LHCII (light harvesting complex II antenna) in stroma-exposed PSII rich thylakoid membranes, to PSI reaction centers. LHCII phosphorylation does not collect light energy for PSI in these cases. (Tikkanen 2012). Only in the margins of the grana thylakoid membrane do LHCII antenna behave according to the traditional view of phosphorylation-induced state transition (Tikkanen 2008). Tikkanen also states that there is substantial evidence to show that the classical mechanism of state transitions is not the sole method for energy balance between the two different photosystem types. There is also evidence to show that LHCII phosphorylation probably connects the regulation of light balance between PSII (photosystem II) and PSI (photosystem I) through unknown non-photochemical quenching mechanisms (Tikkanen 2012), that work not only with PSII but also PSI (Tikkanen 2010). It was also noticed that when light intensity is increased, the PsbS protein is protonated, turning the LHCII antenna into a dissipative state for PSII. (Li 2004, Tikkannen 2012). At lower light levels, LHCII activity is

restored, and PSII activity is increased. Furthermore, phosphorylation is controlled by the enzymes STN7 and STN8 kinases and their opposing phosphatases, that are in turn, closely controlled by light intensity. These kinase functions are completely synchronized with PsbS and the xanthophyll cycle (Tikkanen 2012).

q_Z - due to an unknown longer term xanthophyll cycle mechanism

In 2010, Nilkens and others used NPQ Arabidopsis mutants to determine that under saturation light conditions, q_T or state transitions did not significantly contribute to fluorescence change and resulting quenching relaxation in the dark. Furthermore, samples were tested at moderate illumination to rule out q_I or photoinhibition as the contributor to this fluorescence change. The changes related to q_Z were complete by 30 minutes. It was proposed that under steady state, saturating light conditions, NPQ should be divided into q_E, q_Z, and q_I.

As described by others, q_E is a process that is created and relaxes in the ten second to two hundred second time frame, and is depends on Δph of the thylakoid lumen, the PsbS protein and zeaxanthin formation. The longer portion of q_E from one hundred seconds to about 200 seconds is limited by zeaxanthin synthesis. Relaxation of q_E is regulate the Δph of the thylakoid lumen.

According to the Nilkens group, the proposed q_Z is created in the ten to thirty minute time frame. Psbs is not involved in q_Z, but is wholly dependant on zeaxanthin formation. Relaxation depends on the re-conversion of zeaxanthin to violaxanthin. It should be stated that the group found a sample that seemed to contradict the other q_Z results; however, they said that is was likely due to a retarded relaxation of a large fraction of q_E and not normal middle range fluorescence relaxation characteristics. The test plant was zeaxanthin devoid mutant npq1.

Photoinhibition, q_I, was shown to form after 30 minutes and was dependant on illumination time, intensity and genotype. It was also found the state transitions, q_T, were not a significant contributor to NPQ at saturating light intensity.

q_M – due to chloroplast migration

Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) used multiple methods, Arabidopsis mutants and wild type Arabidopsis plants to determine that the fluorescence change, previously thought to be the result of state transitions, or more recently, thought to be a longer lasting xanthophyll cycle process, was caused, by chloroplast migration. They found, as others have, that under high light levels, chloroplasts move from the top of cells to the sides of cells, partially shading other chloroplasts. This was verified with light microscopy using samples treated to prevent migration after high light migration. They also found that leaf transmittance increased and therefore leaf absorptance was lowered with chloroplast migration. The research concludes that the cause of q_M is a decrease in light photon absorption which creates lower fluorescence yield, rather that a true quenching process. This is thought to be another avoidance process to protect leaves from high light levels. They found that the time scales for adjustment and fluorescence intensity change mirrored the previously used q_T, but extended up to 35 minutes with some plants. Chloroplast migration has been known and studied for a while, and it was stated by Brugnoli in 1992 that chloroplast migration affected chlorophyll fluorescence. The Cazzaniga paper is the first to name chloroplast migration as the source of the q_T and q_Z fluorescence change.

Researchers found that high white actinic light was more effective than high red actinic light at inducing the photoprotective functions of q_M. Chloroplast migration was shown to be controlled by high blue light intensity (Kagawa T. 2001). Arabidopsis mutants that were devoid of q_E (npq4) were tested and Arabidopsis mutants devoid of q_E and chloroplast migration (npq4 photo2) were also tested along with other mutants. In addition, targeted reverse genetic analysis was used to eliminate other contending possibilities by creating a series of double and triple mutant that covered the remaining spectrum of mechanisms that affected chlorophyll fluorescence, including; the composition of various parts the photosynthetic apparatus, carotenoid biosynthesis, and state transitions as sources of q_M.

In regard to q_T being replaced by q_Z, it was found that by using mutants devoid of q_E and zeaxanthin, that the magnitude of q_M did not change but the recovery time in the dark was longer. Plants were grown at 150 μmol photons m^{-2} s^{-1}, and tested at 400 μmol photons m^{-2} s^{-1}, 800 μmol photons m^{-2} s^{-1}, and 1,200 μmol photons m^{-2} s^{-1}. The adjustment time for q_M ranged up to 35 minutes for some mutants.

The Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) observation that the stn7 mutant , devoid of state transitions, has NPQ measurements very similar to the wild type in Arabidopsis, casts strong reservations in regard to the relevance of q_T in Arabidopsis.

The fact that higher intensity white or high intensity blue actinic light is required to activate q_M, or chloroplast migration, indicates the need for either a white light source or a high intensity blue light and red light instead of using a high intensity red and lower intensity blue actinic light (Cazzaniga 2013). This new research shows that measuring artifacts are possible when using a low intensity blue light source for chlorophyll fluorescence measurement. ETR or J, Y(II) or F_{PSII} , NPQ, g_M, C_C and q_I may all include measuring errors without the a reliable light source. Furthermore, all gas exchange measurements contain an error if blue light intensity is not high enough to allow chloroplasts migrations to occur for a specific high light level.

This also may change the times required for proper dark adaptation measurements, and the time to reach steady state photosynthesis under light adapted conditions. Until now, Maxwell K., Johnson G. N, (2000) has been the most sighted paper for reliable steady state photosynthesis conditions at any given light level. It lists 15 to 20 minutes as the time required for 20 wild land plants to reach steady state photosynthesis. Prominent researchers, Lichtenthaler (1999) and Ruban (2009), list the dark adaptation time required for quenching relaxation of q_T at the same 15 to 20 minutes. With this new evidence from Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013), it shows that dark adaption times and the time to reach steady state lighting conditions should be extended, at least at higher light levels. They show that it takes from 20 minutes to 35 minutes for chloroplast migration to adapt to higher light levels and for relaxation in the dark (Cazzaniga 2013). Current research will likely provide some additional surprises in this area, moving forward.

Photoinhibition

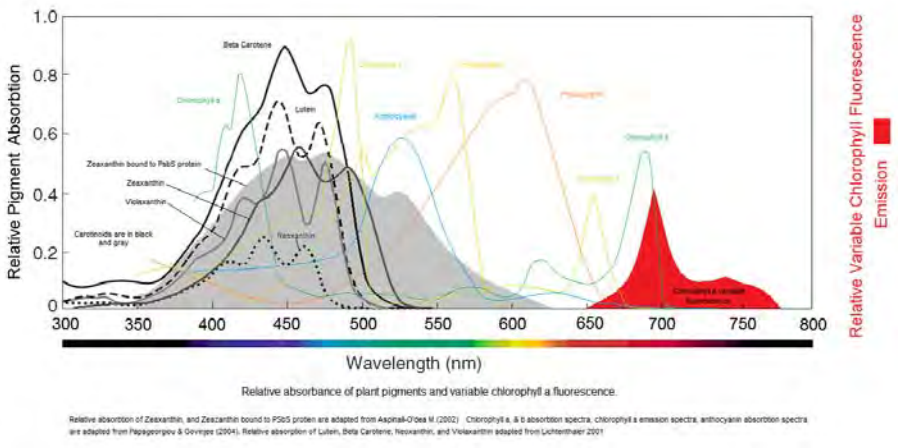
Photoinhibition is a process that can occur at high light levels for extended periods of time. It was thought that the effects of high light levels for shorter periods of time, an hour or two, was a form of acute photoinhibition could be reversed with 20 to 30 minutes of dark adaption (Theile, Krause & Winter 1998). Recent research indicates that this is more likely due to chloroplast migration due to the relaxation times tested (Cazzaniga 2013). Chronic photo-inhibition, caused by several hours of high light exposure, starts to relax or repair at about 40 minutes and may take 30 to 60 hours to fully relax or repair under dark adaptation (Lichtenthaler H. & Babani F. (2004) (Theile, Krause & Winter 1998). When making longer quenching and quenching relaxation parameter measurements related to photo-inhibition and photo-damage mechanisms that are common in chronic high light stress, high heat stress, cold stress and over wintering stress, one should understand that it can take days for full relaxation or repair of the non-photochemical quenching parameters, q_I , to pre-stress conditions. To get an accurate control value for Fm and Fo under chronic photo-inhibition conditions (components of non-photochemical quenching parameters) it is common to dark-adapt for a full night, or 24 hours. (Maxwell and Johnson 2000). To study photoinhibition it may make sense to partially shade samples for a few days before testing to ensure that all residual photoinhibition has relaxed or repaired. It is expected and accepted that there is some residual unrelaxed photoinhibition or NPQ in field plants using dark adapted samples, after sunny days, in the summer time. For this reason, it is important to compare samples with similar light history (Light history application note). Never compare any of the non-photochemical quenching value in samples that do not have the same F_V/F_M because F_V/F_M is the yard or meter stick used to determine these values. For more information on quenching measurements, refer to the OSI application note on quenching measurement.

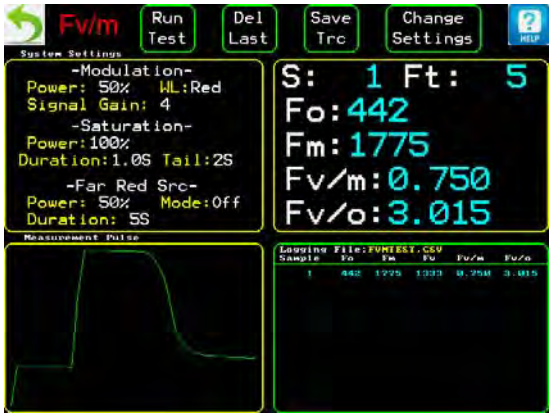
Other plant pigments

Carotenoids are present in the antenna systems and reaction cores and in other locations. They include alpha and beta carotene, and xanthophylls: lutien, zeaxanthin, violaxanthin, antheraxanthin and neoxanthin. Carotenoids are involved in a number of processes including: Acting as antenna in the transfer of energy to PS II and PSI reaction centers; the xanthophyll cycle is used in plant photo-protection to dissipate excess light energy, the blocking of free oxygen and organic radical, and the quenching of chlorophyll excited states as well as singlet oxygen (Gitelson 2002).

The ratio of red light leaf absorption to blue light leaf absorption changes with actinic light intensity level (Bernacchi 2002), (Cazzaniga 2013). There is significant evidence to show that violaxanthin converts to zeaxanthin as light levels increase, and that zeaxanthin works in conjunction with the protein PcbS in a photoprotective role that shifts the absorption spectrum (Aspinall-O'Dea 2002). In addition, zeaxanthin, in stoma guard cells, has been linked to stoma aperture size and appears to be mediated by blue light Kinoshita (2001) Ziegler (1998).

The function of anthocyanins in leaves, has been the subject of debate. They have been found most commonly in cell vacuoles but may be found in all plant tissues. There is evidence to show that they function as longer term photo-protective mechanisms, help serve in the protection of shade leaves from high intensity sunflecks, help provide protection against UV-B, function in antioxidant activity, act as attractors to animals for pollen and seed distribution, or act indirectly in signaling mechanisms involved in plant growth, and development, plant stress response, and gene expression. The affects can vary from one species to the next (Gould 2009).





F_V/F_M measuring screen

F_V/F_M Protocol (or $F_{V/M}$ on the screen) – Background

Dark adapted test - a measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centers were open. 0.79 to 0.83 is the approximate optimal value for most plant species with lowered values indicating plant stress. (Maxwell K., Johnson G. N. 2000) (Kitajima and Butler, 1975). F_V/F_M has a photochemical component and a non-photochemical component (Baker 2004). F_V/F_M is a fast test that usually takes less than two seconds. However, when using pre – illumination with far red light, the test can take between seven to twelve seconds. (see the section on dark adaptation in chapter 1, and refer to our Desk top plant stress guide for a review of its value and limitations.
The assumptions are that before the test, PSII is fully oxidized and quenching mechanisms have relaxed to a known dark adapted level. With the F_V/F_M test, it is important to dark adapt the sample being tested to get reliable measurements. If dark adaption is not done properly, F_M will be lower than it should be, F_o will be higher than it should be and F_V/F_M will be lowered as well. F_V/F_M dark adaption times can vary due to light history and the goal of the measurement. With recent research regarding q_M or chloroplast migration, relaxation of chloroplast migration can take between twenty and thirty five minutes (Cazzaniga 2013). Some scientists only work with pre-dawn dark adapted samples. For more information on dark adaptation , see the special section on dark adaptation.

Non-Photochemical Quenching normally lowers F_V/F_M when a plant is exposed to illumination. By dark adapting, one is allowing the re-oxidation of PSII and the relaxation of NPQ. Experiments should be done on plants to be tested to find the appropriate dark adaption times for best results. If that is not possible, then 35 minutes is a safe time for samples with similar light histories, and where the measurement of photoinhibition is not the goal.

A series of 10 dark adaption white clips are provided with the system to be used for dark adaption measurement. A Clip should be placed on the leaf with the black slider covering the cylindrical opening. After dark adaption, the end of the fiber optic bundle should be placed in the cylindrical opening and the dark slide of the clip should be opened allowing the sample to be exposed to the fiber optic bundle. Dark shrouds can also be used for dark adaption or lights can be turned off in a windowless environment.

To trigger a measurement, either the "red button" on the fiber bundle must be pressed, or the "Measure" button on F_V/F_M measuring screen.

A Cookbook Checklist before making reliable F_V/F_M and OJIP measurements

Accuracy is the ability to hit the bull's eye. In many types of measurements, accuracy is determined by calibrating to a measuring a standard that is traceable to the National Agency. With such measurements, tolerances are always involved.

Repeatability is the ability to achieve the same measurement again and again to a certain tolerance level.

A Reliable measurement is one that is accurate and repeatable.

With Chlorophyll fluorometers, accuracy is determined by following recommended methods and understanding the limitation of the measurement.

F_V/F_M & OJIP

The biggest advantage of F_V/F_M is that it is a measure of PSII performance that puts all samples in the same known dark adapted state before measurement. F_V/F_M is a normalized ratio that does not use a traceable standard. Instead, it's accuracy is determined by properly using the instrument and following the lessons learned about plant physiology by several great researchers. For most species, the optimal F_V/F_M reading for stress free plants is in the range of 0.79 to 0.83 (Maxwell and Johnson 2004). It is important to know that the check list for OJIP is the same as for F_V/F_M. The only difference is light intensity. As long a saturation light source is high enough to saturate samples it will work just fine for F_V/F_M. *However, with OJIP, the intermediate fluorescence values for K, J, and I, along with parameters that use these values in their calculation, can change with light intensity (Vredenberg 2011). For that reason, it is important to use the same intensity every time. In the past 3,000 µmols was used in the Strasser OJIP protocol, more recently 3,500 µmols is used. The OS5p+ comes with the actinic light set at 3,500 µmols; however, it may be set up to 5,800 µmols.*

To get a reliable measurement, one has to follow tested guidelines.

1. Dark-adapt properly knowing the plant's light history. It takes only a few minutes for the xanthophyll cycle and the Δph of the thylakoid lumen to return to a dark-adapted state. (State transitions however, take between fifteen to twenty minutes (Ruban 2009) (Lichtenthaler 1999) . These times can vary somewhat in field plants, and can take slightly longer. At high light levels found in the field, chloroplast migration takes between 20 minutes and 35 minutes to fully relax (Cazzaniga 2013). Deactivation of Rubisco in the dark, takes between 12 -18 minutes in vascular

plants and from 9 minutes to 28 minutes in some photoplankton (MacIntyre 1997). In addition, field plants and other plants that have been exposed to photoinhibition conditions for a number of hours, will retain a certain amount of NPQ for up to 60 hours (Lichtenthaler 2004).This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into most summer field measurements of Fv/Fm. This is all right as long as samples with similar light history are compared. Light history should always be taken into account when comparing samples. It is common for researchers to choose dark adaptation times anywhere from twenty minutes to overnight, using pre-dawn values. Shorter times may be used to study the effects of plant mechanisms. For more information contact OSI for the Dark adaptation application note. (These guidelines are different for quenching measurements and for Rapid Light Curves.)

2. Modulation light intensity setting $F_V/F_M = (F_M-F_O)/F_M$. Minimum fluorescence or F_O, is a dark adapted value measured by exposing the leaf antennae to a very low intensity modulated light before any Q_A has been reduced by an actinic light source. The intensity must be set properly to allow detection, but not high enough to drive any photochemical reduction of Q_A. If it is set too high, it will drive photochemical reduction of Q_A, and provide an F_O value that is too high. When setting the modulating light intensity, the Ft value or fluorescence signal should not rise over a 15 to 20 second period when a leaf is used. If it does, the intensity must be lowered. *OSI now offers an automated modulated light set up routine for its new OS5p+.*

3. Shade leaves vs. Sun leaves. – The F_V/F_M ratio will be slightly higher on sun leaves than on shade leaves (Lichtenthaler 2004).
4. F_V/F_M will be higher with a white saturation pulse than a red saturation pulse.
Some fluorometers use a red saturation pulse. This is not an issue for comparative measurements of plant stress with similar instruments, but values measured on a fluorometer with a white saturation pulse should not be directly compared to measurements of a fluorometer with a red saturation pulse. There is evidence to show that systems with a red saturation pulse correlate but measure consistently lower than systems with white light saturation lights. (Cessna 2010)
5. Maximum F_V/F_M values vary with species. The average maximum F_V/F_M value is between 0.79 - 0.83 (Maxwell and Johnson 2000).
6. Compare samples with a similar light history. Field plants should only be compared to field plants with a similar light history and green house plants should be compared to green houseplants with a similar light history. Due to the fact that it can take up to 60 hours for chronic photoinhibion to relax, photoinhibion can be involved in some measurements more than others. (Lichtenthaler 2004)
7. It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants (Reuter and Robinson 1997)
8. The duration of the saturation pulse should be between 0.5 seconds and 1.5 seconds for higher plants, and 25 to 50 milliseconds for Phytoplankton and cyanobacteria. (Schreiber 1995). Times outside these ranges increase the error in F_VF_M measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity. Longer durations create a form of saturation pulse NPQ that rounds the tail end of the pulse maximum value, and reduces the average maximum saturation pulse value. Opti-Sciences provides a moving 25 ms average to determine the highest FM. This ensures that a reliable value will be measured even if the saturation pulse width or duration is too long.

9. Saturation pulse intensity. Dark adapted leaves saturate easily with lower saturation pulse intensities. It may take a few hundred :mols to saturate shade leaves and sun leaves will saturate

below 1,500 :mols. Lower values may not fully saturate PSII, and provide an error. Higher values always work with dark adapted samples. (Ralph 2005) (Requirements are different for Y(II).)

10. Some F_V/F_M fluorometers have the ability to pre-illuminate dark adapted leaves with far-red light. When this feature is used for five to ten seconds before an F_V/F_M measurement takes place, it activates PSI, and ensures that all electrons have been drained from PSII before the measurement of F_O. While this feature ensures that PSII is completely

re-oxidized, it does not relax the xanthophyll cycle, chloroplast migrations, state transitions, or photoinhibition. Time is still required in a darkened environment to relax all forms of NPQ and to obtain a reliable FV/FM measurement. (Maxwell and Johnson 2000) (Cazzaniga 2013)

11. Fluorescence heterogeneity, or patchy leaf fluorescence, presents itself as different F_V/F_M measurements on different parts of the leaf. It has been found to occur under cold stress conditions, with biotic stress, and under drought stress conditions. By using multiple measurements a sampling plan and averaging, heterogeneity can be over come (Buschmann C. in correspondence by e-mail 2008). Imaging fluorescence can also be used).
12. Part of the minimum fluorescence, the F_O parameter, in F_V/F_M $(F_M - F_O)/F_M$), contains PSI fluorescence as well as PSII fluorescence. With $F_V/F_{M,}$ one is trying to measure the maximum variable fluorescence of PSII in a dark-adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces an error. In C_3 plants, about 30% of F_O fluorescence is due to PSI, and in C_4 plants about 50% of F_O fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in Fm in C_3 plants, and about 12% of Fm in C_4 plants (Pfundle 1998). This not a problem when comparing F_V/F_M measurements for plant stress because PSI fluorescence does not change. It remains constant, and F_V/F_M is a normalized ratio.

There are fluorescence solutions and assays available that are sensitive to most types of plant stress. F_V/F_M is not as sensitive as Y(II) for many types of plant stress. F_V/ F_M is not a sensitive test for drought stress, heat stress, nitrogen stress, nickel stress, sulfur stress, zinc stress, some herbicides and salt stress in some types of plants (Opti-Sciences Plant Stress Guide 2010). It can be used effectively in most other types of plant stress. For specific research results on specific types of plant stress, see the Plant Stress Guide offered by Opti-Sciences Inc.

Y(II) measuring screen

Y(II) -Quantum yield of PSII – an in depth discussion of the value and limitations.

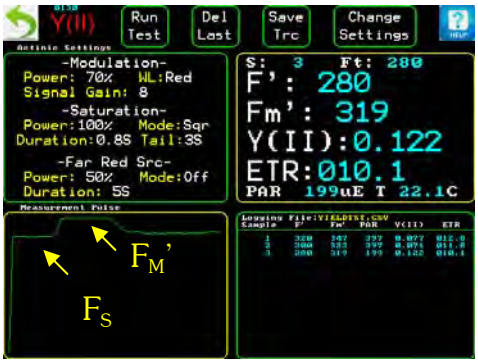
Y(II) or $\Delta F/F_M'$ or $F_M' - Fs / F_M'$) is a time tested light adapted, normalized ratio parameter that is more sensitive to more types of plant stress than F_V/F_M according to a survey of existing research. While F_V/F_M is an excellent way to test for stress and the health of Photosystem II, Quantum Photochemical Yield is a test that allows the measurement of the efficiency of the overall process under actual environmental and physiological conditions.

Quantum Yield of PSII is a measurement ratio that represents achieved efficiency of photosystem II under current steady-state photosynthetic lighting conditions. (Genty 1989), (Maxwell K., Johnson G. N. 2000), (Rascher 2000). It is affected by closure of reaction centers and heat dissipation caused by non-photochemical quenching (Schreiber 2004). It is also affected by chloroplast migration (Cazzaniga 2013).

As ambient light irradiates a leaf, about an average of 84% of the light is absorbed by the leaf, and an average of 50% of that light is absorbed by the antennae associated with PSII and transferred to PSII (Photosystem II) reaction centers. (Leaf Absorption can range from 70% to 90% (Eichelman H. 2004) and PSII absorption can range from 40% to 64% (Edwards GE 1993) (Laisk A. 1996)). Under normal non-stressed conditions, most light energy is channeled into photochemistry with smaller amounts of energy channeled into heat and fluorescence. In photosystem II, this process is competitive so that as plant stress occurs, mechanisms that dissipate heat, photo-protect the leaf, and balance light between photosystem II and photosystem I, change the output of fluorescence and heat. In other words, conditions that maximize photochemistry minimize fluorescence and heat dissipation and conditions that maximize fluorescence minimize photochemistry and heat dissipation.

Once these mechanisms have achieved an equilibrium at a specific light level and temperature, steady state photosynthesis has been achieved a process that was thought to take fifteen to twenty minutes (Maxwell and Johnson 2000), can take 20 to 35 minutes at high light levels found under field conditions. This extended time is due to the time required by different plants to complete

chloroplast migration (Cazzaniga 2013). At this point, a modulated fluorometer can be used to expose a plant to a very intense short light pulse called a saturation pulse. It is designed to momentarily close or chemically reduce all capable PSII reaction centers currently being used in photochemistry while at steady state photosynthesis. Apart from the known exceptions listed under "Correlation to Carbon Assimilation" later in this discussion, quantum photochemical yield will reflect changes in the function levels of PSII antennae, PSII reaction centers, electron transport, and regulatory feedback mechanisms.



$$Y(II) = (F_M' - F_S) / F_M'$$

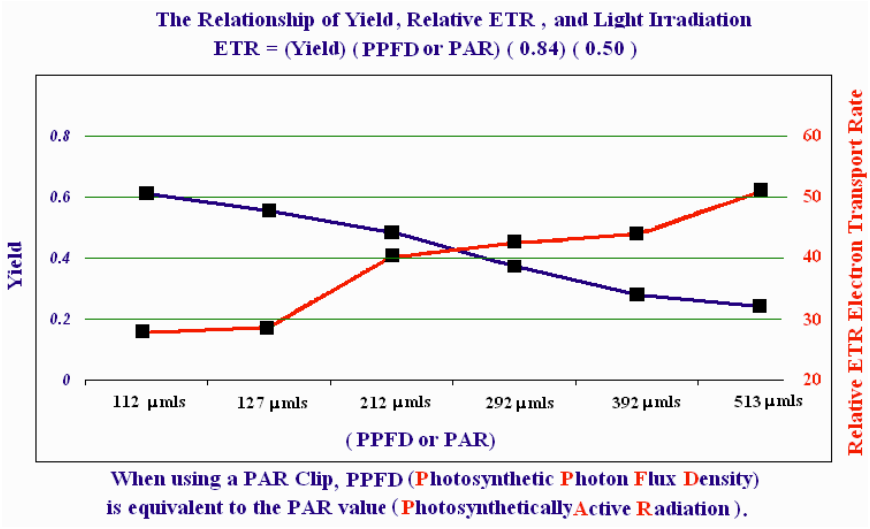
Quantum yield of PSII is measured only at steady state photosynthesis.
F_s is the fluorescence level at steady state photosynthesis, and F_M' maximum fluorescence value measured during a saturation pulse, and is taken to mean that all PSII reaction centers are closed or photo-chemically reduced. In a high light environment, this may not be true and the Multi-flash method may be required. See Multi-flash for more details on pages 71-74.

Y(II) will change at different light levels and temperatures so it can be of great value to use a heavily recommended accessory called a PAR Clip that measures yield relative to light intensity or irradiation level and temperature. PAR Clips measure Photosynthetically Active Radiation between the wavelengths of 400 nm and 700nm. When the dimensions per square meter per second in micro-mols or micro-Einstein's are added, this parameter becomes Photosynthetic Photon Flux Density (or PPFD) (micro-mols and micro-Einsteins are equivalent, and when using a PAR Clip, PAR and PPFD are equivalent). NOTE: It is possible to misinterpret results if PAR and temperature changes are not taken into account. One leaf may appear to be stressed compared to another when the only difference is light irradiation level. PPFD or PAR must be measured very close to the sample or errors can result.

In addition, it is important not to change the orientation of a leaf and to avoid shading the sample measuring area with the PAR clip or by other means. Extraneous reflections and breathing on the sample should also be avoided (Rosenqvist and van Kooten 2006).

PAR Clips also allow measurement of relative ETR or relative Electron Transport Rate. ETR is a parameter designed to measure the electron transport rate of PSII. It has also been found to correlate well with CO_2 assimilation. More advanced fluorometers provide built-in illuminators for greater experimental control of light irradiation intensity. This allows pre-illumination with a controlled predetermined intensity value for sample comparison. The OS5p+ maintains a stable built-in actinic light intensity when used with the PAR Clip.

For reliable Yield and ETR measurements, photosynthesis must be at steady state and with illumination on the same side of the leaf that is being measured (see number eight under correlation to carbon assimilation). Steady state photosynthesis is an equilibrium condition reached after a several minutes of exposure to existing light radiation conditions. Maxwell and Johnson (2000) tested 22 different species of British plant and found that steady state occurred in fifteen to twenty minutes in the plants measured. Recent work done by S. Cazzaniga (2013) indicate that under high light conditions, it can take between twenty minutes to thirty five minutes to reach steady state photosynthesis due to chloroplast migration. (See the application note on q_M and its ramifications for chlorophyll fluorescence on our web site.) Measurements taken under variable lighting conditions may not provide reliable Y(II) results (Rascher 2000). No dark adaptation is required for Y(II) measurements.



Correlation to Carbon assimilation:

In 1989, Genty developed the yield measurement and provided strong evidence of a linear correlation between Yield measurements, Electron Transport Rate, and CO_2 assimilation for C_4 plants (Baker and Oxborough 2004) and many others have confirmed the relationship (Edwards and Baker 1993), (Krall and Edwards 1990, 1991), Siebke 1997). It was found that a curve-linear correlation between Yield and CO_2 assimilation exists for C_3 species where photorespiration can also use significant products of electron transport (Genty 1990), (Harbinson 1990), (Baker and Oxborough 2004). Psydo-cyclic electron transport and other electron sinks may also be involved.

A Cookbook Checklist for making reliable Y(II) measurements – The Limitations of Y(II)

The strong relationship between Y(II) and CO_2 assimilation correlation has been reaffirmed repeatedly by many researchers with the following caveats:

1. At high actinic light levels, the correlation between ETR and CO_2 assimilation breaks down. Y(II) can show an error of up to 22%, and ETR can show an error of up to 41% (Loriaux 2006). It is thought by some to be caused by the inability of the most intense saturation light sources to completely close all PSII reaction centers under high light stress conditions. To compensate for this issue, Earl (2004) uses saturation pulses at various levels and extrapolates the results of a saturation pulse at infinity using linear regression analysis. Loriaux (2006, & 2013) develops a single multiple phased saturation flash with least squares linear regression analysis to correct for this error. In the latest refinement of the process, Loriaux (2013) found that using a saturation intensity of 7,000 μmols to 13,000 μmols, a 20% intensity down ramp, and a ramp rate less than 0.01moles $m^{-2}s^{-2}$ provided the most accurate results. This method restores the correlation of ETR and CO_2 assimilation and it is an option called Multi-flash that is offered On the Opti-Sciences OS5p+ and the OS1p.
2. There is small percentage of chlorophyll fluorescence that comes from photosystem I that does not change with light intensity (PPFD) or plant stress. Therefore, the error is greatest at very high light levels when yield is minimized and PSI fluorescence remaining constant. This error is not large (Baker Oxborough 2004).
3. "Super-saturating flash" error is produced by using a very intense saturation light source that is longer that 2 ms causing multiple turnovers of primary PSII receptor Q_A and the reduction of plasotoquinone to plastoquinol. This raises F_M' and can cause an overestimate of Yield by less than 10% (Baker and Oxborough 2004), (Schreiber 2004). Use of a super-saturation flash is by far the most common method of measuring Y(II) in higher plants.
4. Cold stress can produce a non-linear correlation with CO_2 assimilation. Electron transport of PSII in cold stressed corn far exceeds the requirements for CO_2 assimilation by more than three to one, indicating that under these conditions other electron sinks are at work. The ratio of ETR (a product of Y(II), PAR, leaf absorption ratio, and PSII absorption ratio) to CO_2 assimilation under cold stress can be diagnostic for cold stress. (Fryer M. J., Andrews J.R., Oxborough K., Blowers D. A., Baker N.E. 1998)
5. The ratio of ETR to CO_2 assimilation can be diagnostic for water stress in C_3 plants. C_3 plants exhibit strong electron transport rates for early and moderate levels of water stress even when CO_2 assimilation has decreased due to water or drought stress. This indicates that there are other electron sinks for electron transport. (Ohashi 2005). This problem of early water stress measurement and detection may be overcome by using the Burke assay (Burke 2010). Y(II) can be used to measure very early water stress (Burke 2007 and Burke 2010).
6. Mangrove leaves growing in the tropics. Here again electron transport rate is more that three times that of CO_2 assimilation. It is believed that this is mostly due to reactive oxygen species as an electron sink. (Baker Oxborough 2004), (Cheeseman 1997)

7. Measurements not taken at steady state photosynthesis can lead to non-linearity caused by state transitions. This error can be in the range of 10% to 30% depending on the organism (Allen and Mullineau 2004). The error can be avoided by allowing plant samples to reach steady state. photosynthesis, a process that takes between fifteen and twenty minutes (Maxwell and Johnson 2000).
8. While linear correlation and curvilinear correlation are possible (Genty 1989), (Genty 1990), (Baker Oxborough 2004), exact correlation between fluorescence ETR and gas exchange ETR is not possible due to the fact that fluorescence comes from only the upper most layers of the leaf while gas exchange measurements measure lower layers as well (Schreiber 2004).
9. In CAM plants, gas exchange measurements are not possible during daylight hours so Y(II) measurements can provide insights into daytime light reactions (Rosenqvist and van Kooten 2006). As illustrated by the exceptions listed above, in some cases …"the relationship between light reactions and dark reactions is not straightforward"… The energy molecules ATP and NADPH can be used for carbon fixation and for photorespiration (Rosenqvist and van Kooten 2006), or light reaction electrons may flow to other electron sinks (Ohashi 2005), (Baker Oxborough 2004), (Fryer M. J., Andrews J.R., Oxborough K., Blowers D. A., Baker N.E. 1998). For this reason, it is not uncommon for authors to differentiate between work done under non-photorespiratory conditions and under photorespiratory conditions (e.g. Earl 2004), (e.g. Genty B, Harbinson J., Baker N.R. 1990).
10. Samples should be compared that have similar light histories. It takes between 40 minutes and 60 hours for chronic photoinhibition to relax or repair. As a result, there is always some residual photoinhibition built into chlorophyll fluorescence measurements after a sunny day in the field. Plants that have a few overcast days may not have this residual photoinhibition built into measurements (Lichtenthaler 2004).
11. Chlorophyll fluorescence heterogeneity or patchy fluorescence occurs under some conditions. Here, a few measurements per leaf using an average is a good solution. It happens during drought stress, cold stress, and under low CO_2 levels. (Correspondence with Buschmann).
12. Y(II) and ETR require steady state photosynthesis (Genty 1989, 1990). New research shows that q_M or fluorescence change due to chloroplast migration takes between 20 to 35 minutes to adjust under high actinic light and it can represent up to 30% of NPQ. For these reasons, it is probably better to use longer times to reach steady state photosynthesis (Cazzaniga 2013).
13. Chloroplast migration (Cazzaniga 2013) is responsible for about 30% of nonphotochemical quenching at high actinic light levels. Chloroplast migration only occurs, as it does in nature, when intense white actinic light or an intense blue actinic light are used. Intense red light has little effect on chloroplast migration.

The Opti-Sciences chlorophyll fluorometer models OS5p+ and OS1p can be used to make Y(II) measurements. Both units accommodate digital PAR Clips. Y(II) is the more versatile fluorescence measuring parameter, but it is best to use a system that offers multiple test parameters for diverse stress applications. While systems that provide true Y(II) measurements tend to cost more than ones that provide just F_V/F_M measurements, they offer greater capability for plant stress measurement. See the Opti-Sciences "Desk Top Plant Stress Guide" for more information www.optisci.com .

Relative Electron Transport Rate

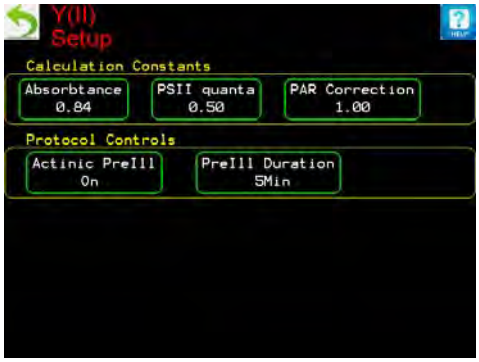
Relative Electron Transport Rate - ETR µmols = (YII) (PAR) (.84) (.5)

ETR or Relative Electron Transport Rate is a parameter that is measured with a PAR Clip. ETR is a relative measurement that provides comparative electron transport rates for PSII at different light or radiation levels. It is considered relative because chlorophyll fluorescence does not correlate exactly with absolute gas exchange measurements. While most fluorescence occurs in the upper most layers of the leaf, gas exchange measures the effects of photosynthesis in deeper layers as well. (Schreiber 2004).

Even with this in mind, relative ETR can be extremely valuable. While exact correlation to gas exchange carbon assimilation is not possible, linear correlation is possible with C_4 plants (Genty 1989) and a curvilinear correlation is possible with C_3 plants (Genty 1990). While four electrons must be transported for every CO_2 molecule assimilated, or O2 molecule evolved, differences from gas exchange measurements can occur under conditions that promote of photorespiration, cyclic electron transport, and nitrate reduction (Schreiber 2004) (Baker, Oxborough 2004). For more detailed information concerning the relationship between fluorescence and gas exchange measurements again refer to Opti-Sciences application note #0509 on Quantum Yield of PSII measurements.

The equation for Relative ETR is ETR = (Y(II) or ΔF/Fm') (0.84) (0.50) (PPFD or PAR)

In this equation, Yield represents overall PSI and PSII yield. It assumes an average leaf light absorbance to be 84%, and the portion of light provided to PSII to be 50%. PPFD is PAR irradiation measured very near the leaf in micromoles or micro-Einsteins (equivalent units). The end result is a close approximation of PSII ETR that can be used for relative evaluation of different samples. With the OS5p+, the actual values for leaf light absorbance and the portion used by PSII can be input into the actual formula to provide more accurate results.



ETR absorption setting screen. This screen can be reached by touching the box with ETR in it, on the Y(II) measuring screen.

The values for leaf absorption and PSII absorption can be changed by touching the green box surrounding the value. For recommended measured values found for different types of plants, refer to the papers listed below.

The PAR Correction factor allows for PAR measurement correction of different types of light sources other than sun light, and the internal actinic light source. Under most conditions, it should be set at 1.00.

It also allows for distance and location error correction capability. When using an artificial light source, the location of the PAR sensor may cause an error relative to the location of the leaf. This correction factor allows following the correction procedure in a paper by Rascher (2000) listed below.

The default values of ETR= (YII) (0.50) (0.84) (PAR) are average plant values used for relative comparisons.

The absolute amounts for leaf and PSII light absorption can vary at steady state with

plant stress, leaf age, chlorophyll content, species and light level. Terrestrial leaf absorbance has been found to vary between 70% to 90% in healthy plants using white light (Eichelman H., Oja V., Rasulov B., Padu E., Bichele I., Pettai H., Niinemets O., Laisk A. 2004), and the percentage of light absorbed by PSII has been found to range from 40% to 60% (Laisk A. and Loreto F. 1996), (Edwards GE and Baker NR 1993).

Relative electron transport rate provides an estimate of CO_2 assimilation. Absorptance conditions. C_4 plants have been found to correlate in a linear manner with CO_2 assimilation. (Genty 1989, 1990). In C_3 plants, Correlation with CO_2 assimilation is curvilinear due to photorespiration, pseudocyclic electron transport or other electron sinks (Fryer 1998),(Genty 1990). Under some forms of plant stress, such as heat stress, and water stress, this relationship can be delayed due to photorespiration and other electron sinks in C_3 plants. There is no significant photorespiration in C_4 plants.

ETR is provided using average values for leaf absorption and PSII absorption. Even so, ETR can provide useful comparative data (U. Schreiber 2004). By plotting ETR vs. PAR, potential ETR rates at maximal quantum yield, photosynthetic capacity, and ETR rate limitations at a given radiation level (light intensity) can be determined. When ETR is graphed vs. PAR at specific leaf temperatures a significant amount of information regarding photosynthesis is obtained. Note: Four electrons must be transported for every CO_2 molecule assimilated or O_2 molecule evolved.

While linear and curve linear correlation with CO2 assimilation is possible, relative ETR does not correlate exactly because while most of radiation is absorbed in the upper layers and provide fluorescent information, some radiation does enter lower layers and the information is not captured in fluorometry. CO_2 and gas exchange carbon assimilation includes information from all layers. (U. Schreiber 2004).

Yield of PSII can vary significantly with light level and with temperature. Without controlling irradiation and temperature it is possible to misinterpret results. In fieldwork where both light and temperature can vary, a lower Yield measurement on one plant as compared to another could be misdiagnosed as stress, when it may only be an increase in irradiation or a change in temperature on the leaf. When a PAR Clip is used to take Yield measurements, the combination can be formidable. Only samples at similar light levels should be compared for plant stress using Y(II).

A PAR Clip is a leaf cuvette that allows the holding of the sample leaf at a repeatable angle and distance from the measuring probe while measuring Yield of PSII, ETR, PAR, and leaf temperature. These values are contained in same data file with a time and date stamp. PAR (Photosynthetically Active Radiation) is irradiated light between the wavelengths of 400 nm and 700nm and it is measured very near the sample measuring area and at the same angle as the leaf. When PAR is measured using a PAR Clip and the dimensions per square meter per second are used, the value becomes PPFD (Photosynthetic Photon Flux Density), (When using a PAR Clip the terms PAR and PPFD are interchangeable).

Some PAR Clips offer the option of using a predetermined light intensity level from an internal fluorometer light source to measure Yield and ETR. In this case, irradiation levels can be forced

to be consistent for each sample. Note: When using internal illumination it is important to allow time for a plant to reach steady state photosynthesis before measurement or errors will result. Even small changes in irradiance level can change the measurement of both yield and ETR. Small changes in light level can cause errors if one does not allow for photo-protective changes, and state transitions. Maxwell and Johnson (2000) found that it takes between 15 minutes and 20 minutes for plants to reach steady state. Recently Cazzaniga (2013) found that it can take between 20 to 35 minutes for chloroplast migration to occur. As a result it may take 35 minutes at a specific light level for steady state photosynthesis to occur at high light levels. (For more information on this topic see Opti-Sciences App note # 0509, “Yield of PSII- Value & Limitations”.)

Some PAR Clips, such as the digital PAR clip for Opti-Sciences OS5p+, allow PAR measurement while an internal light source is being used, thus providing a reliable PAR or PPFD values over time. The OS5p+ provides a stable pre-illumination light intensity when it is used with a PAR clip. There is enough reserve intensity to maintain 1,800 μmols for long periods of time.

Quenching measurements, an overview

(Images from the OS5p+)

Kramer Lake Model & Hendrickson-Klughammer Lake Model

Quenching measurements and understanding the quenching fluorescence curve:

Introduction:

This article is an overview of the value and limitations of kinetic traces and provides a basic understanding of photochemical and non-photochemical quenching measurements. The puddle model, the Kramer lake model, and the Hendrickson lake model parameters that include NPQ resurrected to the lake model by Klughammer & Schrieber will be reviewed. Practical considerations are added to the discussion.

Quenching traces are used in measuring photo-protective mechanisms, state transitions (there is now some evidence that change in fluorescence thought to be due to state transitions may be due to chloroplast migration in the cell), photoinhibition, and passive energy dissipation. Most of the quenching parameters require steady state photosynthetic conditions for reliable results; however, Klughammer states that Y(NO) is not limited to steady state measurement. Without a good understanding of the mechanisms that affect the light trace, machine artifacts and user errors can be included in measurements.

Chlorophyll fluorescence signal:

Variable chlorophyll fluorescence has been found to provide significant information regarding the light reaction of photosynthetic processes, plant health, and plant stress measurement. Variable chlorophyll fluorescence from light that is absorbed by PSII has been shown to vary with these conditions and allow measurement, where as PSI fluorescence is low, and does not variable. The fluorescence signal that comes from PSII is the result of a competitive process with photochemistry and heat dissipation (heat dissipation can further be divided into non-radiation decay and photo-protective regulated heat dissipation). For example, when most of the light is used by photochemistry, less is given off as fluorescence and heat. PSII has also been found to be sensitive to most types of plant stress (Baker 2008).

Actinic Light Source

For quenching measurements, it is recommended that the PAR clip be used with a shroud and tripod for quenching measurements and for the creation of light curves and rapid light curves. The PAR clip may also be used without a shroud for pre-dawn measurements or in a darkened room. When the PAR clip is used with a dark shroud, the actinic light remains at a stable intensity during the entire quenching measurement. There is enough reserve intensity to maintain up to a 1,800 µmol value for long periods of time.

Why do it this way? Most built-in fluorometer light sources used as actinic sources for quenching measurements, and light curves, decline in intensity during these measurements. This is due to the fact that heat from the internal light sources reduces light output. It can happen to halogen light sources and to LED light sources. When this happens, the photosynthetic sample may never really

reach steady state photosynthesis, a process that takes between fifteen and twenty minutes at a specific light level (Maxwell and Johnson 2000). Such light sources can produce errors in all quenching parameters including Y(II).

Lake model and puddle model quenching parameters.

Understanding of the organization of antennae and reaction centers has changed over the years. It is now understood that a single antennae does not link only to a single reaction center as was previously described in the puddle model. Current evidence indicates that reaction centers are connected with shared antennae in terrestrial plants. q_P, the parameter that has been used in the past to represent the fraction of PSII reaction centers that are open, is a puddle model parameter. Dave Kramer (2004) has come up with a set of fluorescence parameters that represent the newer shared antennae paradigm called the lake model.

Others have also come up with more simplified equations that eliminate the need for the measurement of F_o and F_o' and approximate the measurements made by Kramer. Hendrickson's (2004) work offered such a solution with Y(NPQ) measurements that are consistently and only marginally lower values than Kramer's work, and Y(NO) measurements that are consistently and marginally lower except at high light levels and low temperatures than Kramer's work. He speculates that the differences in values between Kramer and his own were possibly due to the difficulties in making F_o' measurements. Furthermore, Hendrickson does not provide a parameter like q_L to estimate the fraction of open PSII centers.

From Hendrickson's work, and earlier works by Cailly (1996) and Genty (1989, 1996), Klughammer and Schreiber derive simplified equations that allow for Hendrickson's parameters, and also allow users to reconcile NPQ puddle model measurements with the lake model.

One will be able to choose Kramer parameters, Hendrickson with NPQ parameters, or puddle model parameters. The use of puddle model parameters have been retained because they allow for the separation of photo-protective mechanisms, state transition measurements, and photo-inhibition in quenching relaxation protocols. There is also a significant volume of work done using the older puddle model parameters that may be valuable for comparison. In addition, the reconciliations of NPQ with lake model by Klughammer and Schreiber allow separation of q_E, state transitions q_T, and photoinhibition q_I with the Hendrickson lake model.

The loss of light energy in the plant due to fluorescence, comes primarily from the PSII reaction. When leaves have been dark-adapted, the pools of oxidation-reduction intermediates in the electron transport pathway return to a oxidized state and quenching mechanisms relax. After dark adaptation, a low intensity modulated light turns on and off and the minimal fluorescence signal, F_o, is measured. The modulate light source is at an intensity too low to drive chemical reduction of Q_A, but high enough allow measurement. Upon saturation illumination of a dark-adapted leaf, there is a rapid rise in variable chlorophyll fluorescent light emission from PSII as all available reaction centers are closed, and the maximum amount of light is channeled to variable chlorophyll fluorescence. Multiple turn-overs of the Q_A molecule occur before maximal fluorescence, F_M.

Quenching equations:

Kramer, and Hendrickson / Klughammer &Schreiber's, lake model parameters account for all light that is absorbed by PSII. All parties agree with the following equation.

Kramer's equation is $1 = Y(II)+Y(NPQ)+Y(NO)$
$Y(II)$ is quantum yield of photochemical energy also known as $)F/F_M'$ or $(F_M'- F_S')/ F_M'$

For comparison purposes, the differences in the equations are listed below.

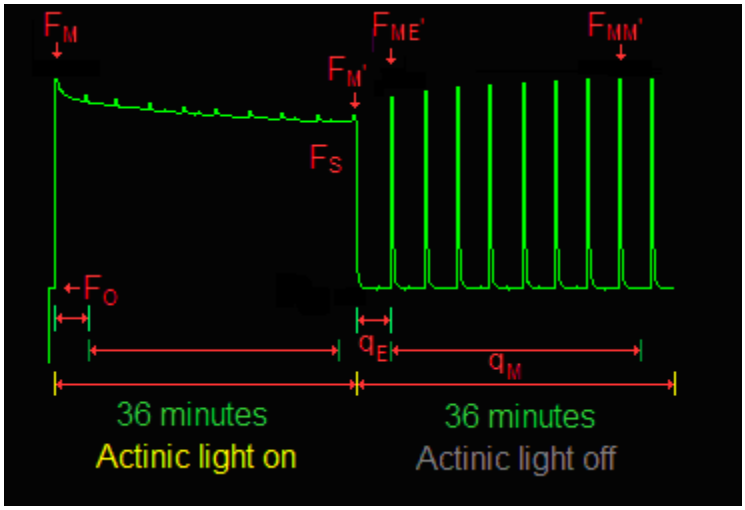
Kramer's equations
$Y(II) = (F_{M'} -F_S)/F_{M'}$ or $\Delta F'/ F_M'$
$q_L = q_P (F_{o'}/F_s)$
$Y(NO) = 1/(NPQ+1+q_L(F_M/F_O -1))$
$Y(NPQ) = 1- Y(II)-Y(NO)$

Hendrickson's equations & NPQ resurrected to the lake model from the puddle model by Klughammer and Schreiber
$Y(II) = (F_{M'} -F_S)/F_{M'}$ or $\Delta F_M'/ F_M'$
$Y(NO) = F_S/F_M$ or F'/F_M
$Y(NPQ) =(F_S/F_{M'})-Y(NO)$ or $(F'/F_M') - Y(NO)$
$NPQ= Y(NPQ)/Y(NO)$ or $NPQ = (F_M-F_M')/F_M'$

Puddle model parameters
$q_P = (F_{M'} - F_S)/(F_{M'} -F_O)$ Above 0.4, F_o' should replace F_o
$q_N = 1- ((F_{M'}-F_O) /(F_M-F_O))$ or $q_N = 1- ((F_M'-F_O) / (F_M-F_O))$ Above 0.4, F_o' should replace F_o
$NPQ = (F_M-F_{M'})/F_{M'}$

Quenching relaxation valid with Hendrickson lake model and puddle model equations.
$NPQ=q_E+q_T+q_I$ or $NPQ = q_E+q_Z+q_I$ or $NPQ =q_E+q_M+q_I$
$q_E = ((F_{ME}-F_{M'})/(F_M-F_{M'}))$ is the relaxation saturation value at four minutes to ten minutes in the dark. (Time is adjustable).
$q_M = ((F_{MM}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at twenty to thirty five minutes in the dark. (Time is adjustable) thirty five minutes is the default value.
$q_Z = ((F_{MZ}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at twenty minutes to 30 minutes in the dark. (Time is adjustable) thirty minutes is the default value.
$q_T = ((F_{MT}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at fifteen to twenty minutes in the dark. (Time is adjustable) twenty minutes is the default value.

$q_I = ((F_M-F_{MM}) / F_M-F_{M'}))$ Relaxation of qI starts at about forty minutes and can take up to sixty hours. qI can be determined from the dark adapted F_M measurement and the saturation pulse at thirty five minutes used for q_M .



Quenching relaxation parameters notes.

Puddle model event times for q_E, q_T and q_I were taken from Lichtenthaler (1999). Descriptions of qE, and qI are taken from Muller P., Xiao-Ping L., Niyogi K. (2001), q_T is taken from Rubin, & Johnson (2009), Allen, Mullineau (2004), and Lichtenthaler (1999) , q_M is taken from Cazzaniga (2013), q_Z is taken from Nilkens (2010)

According to Baker (2008), the event times such as the time it takes for q_E, used in the quenching relaxation test, can change under field conditions. Values of two minutes to seven minutes are reported by Baker (2008).

Definitions - Lake Model Parameters

Y(NPQ) is a lake model quenching parameter that represents heat dissipation related to all photo-protective mechanisms also called regulated heat dissipation. (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004) (Klughammer and Schreiber 2008).
A low Y(NPQ) at high light levels is an indication of sub-optimal photo-protective mechanisms. (Klughammer and Schreiber 2008).

Y(NO) is a lake model quenching parameter that represents all other components of non-photochemical quenching that are not photo-protective. They include non-radiative decay, and fluorescence. Part of Y(NO) includes photoinhibition (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004). Klughammer and Schrieber define Y(NO) as the "fraction of energy that is passively dissipated in the form of heat and fluorescence mainly due to closed PSII reaction centers". Hendrickson calls Y(NO) constitutive heat dissipation. A high Y(NO) value after dark adaptation is an indication of photo-damage. (Klughammer and Schreiber

2008). According to Klughammer (2008), Y(NO) is the only quenching parameter that does not need to be taken as steady state photosynthesis.

q_L is the lake model quenching parameter that represents photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004). It is available in the Kramer equations but not the Hendrickson – Klughammer equations.

Y(II) = quantum yield of photosynthetic energy. The equation is the same as for $\Delta F/F_M'$. $(F_M' - F_S) / F_M'$

Puddle model parameter reconciled with the lake model
(Klughammer and Schreiber 2008)

NPQ = Y(NPQ)/Y(NO) or NPQ= $(F_M-F_M')/F_M'$ Klughammer and Schreiber reconcile NPQ with the lake model using simplified parameters.

NPQ (resurrected puddle model parameter valid in Klughammer simplified Lake model equations. See above.) is non-photochemical quenching and is a measure of heat dissipation. NPQ is an alternate expression of non-photochemical quenching. It provides an estimate of quenching without knowledge of F_O'. The advantage of NPQ over q_N depends on the specific application. NPQ is more heavily affected by non-photochemical quenching that reflects heat-dissipation of excitation energy in the antenna system. So, it may be thought of as an indicator of 'excess excitation energy'. Alternatively, NPQ is relatively insensitive to the part of nonphotochemical quenching associated with q_N values lower than 0.6. The range of NPQ is affected by ph of the thylakoid lumen, which is an important aspect of photosynthetic regulation, state transitions and photoinhibition. Numbers range from zero to infinity with typical samples measuring in the 0.5 to 3.5 range (Maxwell and Johnson 2000). (Bilger & Björkman, 1990), (Muller P., Xiao-Ping L., Niyogi K. 2001).

Definitions - Puddle Model Parameters

NPQ (puddle model parameter) is non-photochemical quenching and is a measure of heat dissipation. NPQ is an alternate expression of non-photochemical quenching. It provides an estimate of quenching without knowledge of F_O'. The advantage of NPQ over q_N depends on the specific application. NPQ is more heavily affected by non-photochemical quenching that reflects heat-dissipation of excitation energy in the antenna system. So, it may be thought of as an indicator of 'excess excitation energy'. Alternatively, NPQ is relatively insensitive to the part of nonphotochemical quenching associated with qN values lower than 0.6. Much of the range of NPQ is affected by Δph of the thylakoid lumen which is an important aspect of photosynthetic regulation, it is also affected by state transitions and photoinhibition. Numbers range from zero to infinity with typical samples measuring in the 0.5 to 3.5 range (Maxwell and Johnson 2000). (Bilger & Björkman, 1990), (Muller P., Xiao-Ping L., Niyogi K. 2001).

q_N (puddle model parameter) is similar to NPQ but requires F_O' in the calculation. q_N is defined as the coefficient of non-photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (F_V) and not the minimal fluorescence (F_O). In cases where q_N is greater than 0.4 this may not be a good assumption. When q_N is above 0.4, F_O' should replace F_O in q_P equations. q_N is less sensitive than NPQ at higher values (Maxwell and Johnson 2000). By using the Far-Red source after actinic illumination is turned off, the PSII acceptors re-oxidized and PSI is reduced. An $F_{o'}$ value is measured and used for corrections to the quenching coefficients. Numbers range from zero to one. (puddle model) (Van Kooten & Snel, 1990)

q_P (puddle model parameter) is the quenching parameter that represents photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. q_P is defined as the coefficients of photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (F_V) and not the minimal fluorescence (F_O). In cases where q_N is greater than 0.4 this may not be a good assumption. When q_N is above 0.4, F_O' should replace F_O in q_P equations. By using the Far-Red source for post illumination, the PSII acceptors may be re-oxidized through the illumination affect on PSI. A new F_O' value may be measured and used for corrections to the quenching coefficients. This assumes the PSI acceptors are properly activated, which may not be the case in a dark adapted sample. Therefore, the F_O' determination should be done after induction of photosynthesis has reached steady state. Numbers range from zero to one. (Puddle model) (Van Kooten & Snel, 1990)

Quenching Relaxation Definitions for Lake and Puddle Model

q_E, q_M, q_Z, q_T, q_I

q_E -(puddle model and lake model parameter) is the quenching parameter that represents the photo-protective mechanisms in the leaf that allow rapid compensation for changes in light levels due to cloud cover and increased light intensity. It is directly related to ΔpH of the thylakoid lumen and the xanthophyll cycle. (Muller P., Xiao-Ping L., Niyogi K. 2001) This process is completed in two to four minutes after an actinic light is turned on but may be as long as seven minutes in field grown leaves (Baker 2008), (Lichtenthaler 1999). It is delineated from NPQ by using a quenching relaxation method. Some researchers in the past have also divided q_N into q_E, q_T, and q_I instead of NPQ (Lichtenthaler 1999) The relaxation characteristics of field plants can vary with changing environmental conditions, for example q_E may take as long as seven minutes (Baker 2008).

q_T -(puddle model and lake model parameter) is not true quenching. Instead, the parameter represents state 1 and state 2 transitions. This value is negligible in higher plants at high light levels but may be substantial at low light levels (Lichtenthaler 1999) (Baker 2008). According to Ruban (2008) state transitions require between fifteen and twenty minutes to complete. It can be delineated from NPQ by using a quenching relaxation method (Muller P., Xiao-Ping L., Niyogi K. 2001). For more information on state transitions, and how they affect fluorescence measurement contact Opti-Sciences for the application note on state transitions. The relaxation

characteristics of field plants can vary with changing environmental conditions (Baker 2008). At near higher light levels and near saturation light conditions fluorescence changes starting before q_E is ended and lasting for between 20 minutes and 35 minutes has been shown to be the result of chloroplast migration or q_M in dicots (Cazzaniga 2013). It is also likely in moncots (Maai 2011).

q_I - (puddle model and lake model parameter) is the quenching parameter that represents photo-inhibition and photo-damage. (Puddle model) (Muller P., Xiao-Ping L., Niyogi K. 2001) According to Lichtenthaler (1999, 2004) chronic photo-inhibition starts to relax after forty minutes in the dark and may take up to sixty hours. It can be delineated from NPQ by using a quenching relaxation method. The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008). There is some residual photoinhibition built into fluorescence measurements on sun leaves after a sunny day in the summer. Light history should be evaluated before comparisons between measurements.

q_Z - due to an unknown longer term xanthophyll cycle mechanism. In 2010, Nilkens and others used NPQ Arabidopsis mutants to determine that under saturation light conditions, q_T or state transitions did not significantly contribute to fluorescence change and resulting quenching relaxation in the dark. Furthermore, samples were tested at moderate illumination to rule out q_I or photoinhibition as the contributor to this fluorescence change. The changes related to q_Z were complete by 30 minutes. It was proposed that under steady state, saturating light conditions, NPQ should be divided into q_E, q_Z, and q_I. As described by others, q_E is a process that is created and relaxes in the ten second to two hundred second time frame, and is depends on Δph of the thylakoid lumen, the PsbS protein and zeaxanthin formation. The longer portion of q_E from one hundred seconds to about 200 seconds is limited by zeaxanthin synthesis. Relaxation of q_E is regulated the Δph of the thylakoid lumen.

According to the Nilkens group, the proposed q_Z is created in the ten to thirty minute time frame. Psbs is not involved in q_Z, but is wholly dependant on zeaxanthin formation. Relaxation depends on the re-conversion of zeaxanthin to violaxanthin. It should be stated that the group found a sample that seemed to contradict the other q_Z results; however, they said that is was likely due to a retarded relaxation of a large fraction of q_E and not normal middle range fluorescence relaxation characteristics. The test plant was a zeaxanthin devoid mutant npq1.

Photoinhibition, q_I, was shown to form after 30 minutes and was dependant on illumination time, intensity and genotype. It was also found the state transitions, q_T, were not a significant contributor to NPQ at saturating light intensity.

q_M – chloroplast migration

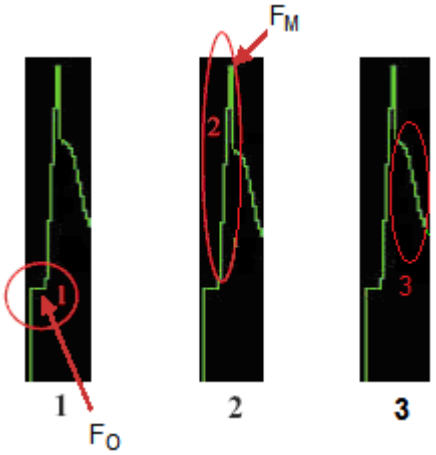
Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) used multiple methods, Arabidopsis mutants and wild type Arabidopsis plants to determine that the fluorescence change, previously thought to be the result of state transitions, or more recently, thought to be a longer lasting xanthophyll cycle process, was caused, by chloroplast migration. They found, as others have, that under high light levels, chloroplasts move from the top of cells to the sides of cells, partially shading other chloroplasts. This was verified with light microscopy using samples treated to prevent migration after high light migration. They also found that leaf transmittance increased and therefore leaf absorptance was lowered with chloroplast migration. The research

concludes that the cause of q_M is a decrease in light photon absorption which creates lower fluorescence yield, rather that a true quenching process. This is thought to be another avoidance process to protect leaves from high light levels. They found that the time scales for adjustment and fluorescence intensity change mirrored the previously used q_T , but extended up to 35 minutes with some plants. Chloroplast migration has been known and studied for a while, and it was stated by Brugnoli in 1992 that chloroplast migration affected chlorophyll fluorescence. The Cazzaniga paper is the first to name chloroplast migration as the source of the q_T and q_Z fluorescence change.

Researchers found that high white actinic light or intense blue light actinic light was required for the light avoidance mechanism, chloroplast migration, to behave as it does in nature. Intense red light did not produce significant chloroplast migration or q_M. Chloroplast migration was shown to be controlled by high blue light intensity (Kagawa T. 2001). Arabidopsis mutants that were devoid of q_E (npq4) were tested and Arabidopsis mutants devoid of q_E and chloroplast migration (npq4 photo2) were also tested along with other mutants. In addition, targeted reverse genetic analysis was used to eliminate other contending possibilities by creating a series of double and triple mutant that covered the remaining spectrum of mechanisms that affected chlorophyll fluorescence, including; the composition of various parts the photosynthetic apparatus, carotenoid biosynthesis, and state transitions as sources of q_M.

In regard to q_T being replaced by q_Z, it was found that by using mutants devoid of q_E and zeaxanthin, that the magnitude of q_M did not change but the recovery time in the dark was longer. Plants were grown at 150 μmol photons m^{-2} s^{-1}, and tested at 400 μmol photons m^{-2} s^{-1}, 800 μmol photons m_{-2} s_{-1}, and 1,200 μmol photons m^{-2} s_{-1}. The adjustment time for q_M ranged up to 35 minutes for some mutants. The Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) observation that the stn7 mutant , devoid of state transitions, has NPQ measurements very similar to the wild type in Arabidopsis, casts strong reservations in regard to the relevance of q_T in Arabidopsis.

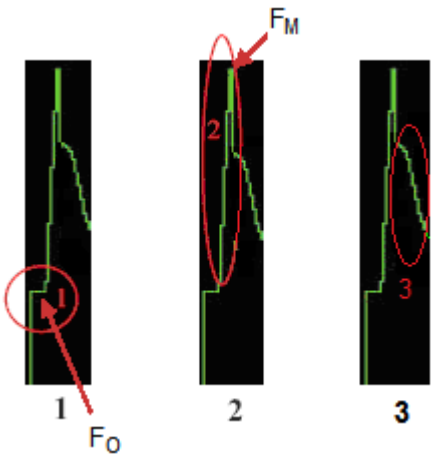
Understanding the Quenching Mode Trace



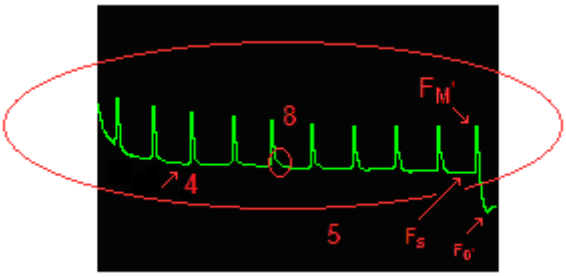
#1 represents a condition that is normally dark adapted (proper dark adaption is required when making nonphotochemical quenching measurements) with the sample shielded from any actinic light that would drive photosynthesis. The only light on at this time is the modulated measuring light at about 0.1umols intensity. This is not enough to drive photochemical reduction of Q_A but it is enough to detect and measure minimum fluorescence from the leaf antennae. In most commercial fluorometers, 30% of the intensity of F_O in C_3 plants is the result of fluorescence from PSI. In C_4 plants, 50% of the intensity of F_O is the result of fluorescence from PSI. This contributes to a small error in F_V/F_M measurements and creates an underestimation of maximum quantum efficiency. Fluorescence from PSI is not variable, it is constant.

#2 shows the first saturation pulse flash. This is a very intense short lasting flash of light that is designed to saturate PSII and close all available reaction centers. For higher plants, the optimal time duration for a saturation pulse has been found to be between 0.5 seconds to 1.5 seconds (Rosenqvist and van Kooten 2005). For Algae and cyanobacteria the optimal duration of the saturation pulse is shorter, 25 to 50 ms (Schreiber 1995). Opti-Sciences uses an eight point rolling average over a 25 ms period to determine maximum F_M and F_M'. This prevents saturation pulse NPQ from causing an error if the saturation pulse duration is set to be too long, and ensures reliable results on land plants or with algae. Furthermore, either square topped saturation flashes or Multiflash may be used during quenching measurements if desired. (See the Multi-Flash section for more details on the protocol). With dark adapted samples, complete closure of all PSII reaction centers can be accomplished with minimal saturation intensity. PAR Light values of 3,000 mols or higher are commonly used. Certainly values of 3000 µmols or higher will fully saturate any properly dark adapted sample.

The rise from F_O to F_M represents multiple turn overs of Q_A, the primary quinone receptor. PSII is fully reduced at F_M with all available PSII reaction centers closed or reduced. At this point, a maximum amount of fluorescence is measured. Most light energy goes to fluorescence at F_M. For more details about F_M, see the review of variable chlorophyll fluorescence in this document.



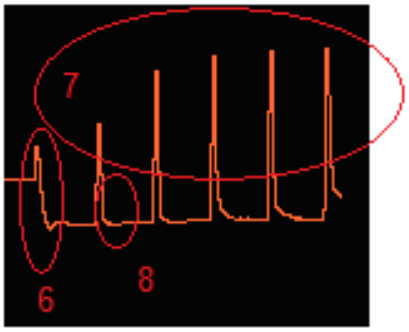
#3
After F_M, the white light LED actinic light source is turned on to the preprogrammed light level. The fluorescence signal competes for energy with photochemistry and heat dissipation. Since photochemistry and heat dissipation mechanisms have just been initiated, most of the energy goes to fluorescence. The fluorescence signal starts to fall as electron transport and carboxylation begins (Schreiber 2004). The photo-protective mechanisms of the xanthophyll cycle and Δ pH of the thylakoid lumen are also beginning to affect the signal to drive down the intensity values of both the saturation flashes and the fluorescence signal itself at higher light intensities. These are heat dissipation mechanisms. The fluorescence signal continues to drop as full activation of Rubisco continues. Full activation of Rubisco in both algae and higher plants takes between three to four minutes (Baker 2008).



#4 -5 In addition, Nonphotochemical quenching, q_E, or a measure of the photo-protective xanthophyll cycle and ΔpH of the thylakoid lumen take between about two minutes to seven minutes to adjust to a new light level (Baker 2008). Field plants take longer that other plants to adjust. #4 is a likely place for the end of q_E adjustment. It is believed that there is an overlap of the initial q_E mechanisms and intermediate plant mechanisms. At low light levels it is still possible that q_T or state transitions overlap with q_E, in plants where they exist. At higher light levels in higher plants, q_M or chloroplast migration is also believed to overlap with q_E (Cazzaniga 2013). At higher light levels that exist in the field, Chloroplast migration is responsible for the fluorescence adjustment that is seen from about 4 minutes to about 20 to 35 minutes (Cazzaniga

2013). This change, as it occurs in nature, can represent up to 30 % of nonphtotochemical quenching when an intense white or an intense blue actinic light are used. There is no significant chloroplast migration under a high red actinic light. This has been shown in dicots, and it is likely also the case in moncots because chloroplast migration also occurs in monocots (Maai 2011). While the mechanisms of chloroplast migration and state transitions are not truly nonphotochemical quenching, they are grouped in NPQ just the same. NPQ includes mechanisms involved in photo-protection, chloroplast migration, state transitions, and photoinhibition. State transitions, where they exist, take between 15 to 20 minutes to adjust. (Baker 2008) Saturation flash intensity values are driven down by non-photochemical quenching, and steady state photosynthesis takes between fifteen and twenty minutes (Maxwell and Johnson 2000). However, it has been found that under high light levels chloroplast migration takes between 20 to 35 minutes to complete (Cazzaniga 2013). Under photo inhibitory conditions D1 protein degradation found in PSII reaction centers close some PSII reaction centers. Other mechanisms have also been suggested for being involved in photoinhibition.

It is common to take quenching measurements at steady state photosynthesis after the leaf has fully adapted to a specific light level. Most light adapted parameters are defined at steady state. However, Klughammer claims that Y(NO) can be measured at other times. Graphing quenching values at non steady state conditions through steady state is done to understand the process. (MacIntyre, Sharkey, Geider 1997) and state transitions take between fifteen and twenty minutes (Ruban A.V., Johnson M.P., 2009), (Allen J. F., Mullineaux C.W., 2004), (Lichtenthaler H. K., Burkart S., (1999). The fluorescence signal and saturation flash intensity are affected by light level, heat and cold, as well as many other forms of plant stress , and the plants ability to deal with plant stress. (see The Opti-Sciences Desk Top Plant Stress Guide for more information www.optisci.com). The saturation (Ruban A.V., Johnson M.P., 2009), (Allen J. F., Mullineaux C.W., 2004) (Lichtenthaler H. K., Burkart S., 1999). qI measurements may take several hours under photoinhibitory light conditions.



6
At this point the actinic light source is turned off, and a far red light is turned on for several seconds to activate PSI and drain all remaining electrons from PSII. This results in a quenched measurement of Fo called Fo', the minimum value measured. F_O' is used in the quenching parameters q_P, q_N, and Kramer's Y(NPQ), q_L, and Y(NO). I has also been used in q_E , q_T , and q_I when q_N is used in place of NPQ. After five to ten seconds, the far red light is turned off. The Far red light at 735 nm is too long to drive PSII, but it will drive PSI.

7

This section of the graph is used for non-photochemical quenching relaxation measurements required in the puddle model and the lake model for separation of q_E, q_M, q_Z, q_T, and q_I. During this phase of the graph, the actinic light is automatically turned off and the sample is in the dark. Only the modulated light and saturation pulses are used here. The increase in the peak height is a result of the relaxation mechanisms grouped into nonphotochemical mechanism including ; photo-protective mechanisms, state transitions (where they exist), q_M chloroplast migration, and eventually photoinhibition. Lichtenthaler found that the relaxation of photo protective mechanisms that involve Δph of the thylakoid lumen and the xanthophyll cycle takes between 2 and 4 minutes. A saturation pulse at the end of this period can be used to measure q_E photo-protective mechanisms (Lichtenthaler 1999). The relaxation characteristics of field plants can vary with changing environmental conditions. He states that q_E may take up to 7 minutes in field plants (Baker 2008).

The relaxation of state transitions takes between 15 and 20 minutes, so a saturation pulse after twenty minutes in the dark can provide a measurement of q_T. A saturation pulse after 20 to 35 minutes in the dark can be used to measure q_M or chloroplast migration. qI or photoinhibition can also be determined with this peak, because F_M is known from the dark adapted first pulse and the difference is considered to be photo inhibition. Chronic photoinhibition starts to relax at about 40 minutes in the dark and Lichtenthaler (1999, 2004) found that it could take up to 60 hours for complete relaxation of photoinhibition. It is common for researchers to dark adapt overnight (Maxwell and Johnson 2000) when making quenching measurements.

It should be noted that NPQ should only be used to compare plants of the same species and with the same Fv/Fm values (Baker 2008), a (Maxwell and Johnson 2000).

In addition, with the reconciliation of NPQ to lake model parameters, it becomes possible to use quenching relaxation with the Hendrickson lake model. It is also possible that at high light levels that there is some overlap between q_E and q_M (Cazzaniga 2013).

It is recommended to use a PAR Clip, a shroud, and a tripod to measure all quenching parameters. This ensures that all measurements are made at steady state photosynthesis at a specific light level. The PAR Clip measures the instrument output, and maintains the programmable light setting.

The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008).

8

After a saturation flash, the tailing off of the signal is the result of NPQ caused by the saturation flash. According to Rosenqvist and van Kooten (2006) it takes between one and two minutes for complete dissipation of saturation pulse NPQ. With this in mind, saturation pulses should be spaced to avoid a build up of NPQ. It is also mentioned that photo-damage can occur to samples when saturation flash intensities are too high in the dark when there is no actinic light. There is evidence that damage does not occur on samples in the light. (Rosenqvist 2006). In some cases we have found that saturation flash NPQ takes longer than 2 minutes, especially during long

quenching protocols with many saturation flashes. If one is concerned, space the saturation flashes to 3 or 4 minutes apart.

More Helpful Hints for Setting Test Variable in Quenching Protocols.

Saturation intensity

The saturation pulse should be intense enough to completely close all PSII reaction centers. To test this, one can try different saturation intensities and examine the saturation peak. This can be done in the Y(II) protocol. After a measurement is made, a partial graph of the saturation pulse is viewed.

One must adjust the intensity high enough so that the most of the top of the saturation pulse is flat. If it is rounded at the middle of the top, more intensity is needed. According to studies done on the subject, even the most intense saturation pulses do not damage plant tissue in a light adapted environment. It has been found that damage can occur with night measurements if the saturation pulses are made too frequently (Rosenqvist and van Kooten 2006) (Albert Porcar-Castell 2008). With this in mind, one can use maximum intensity for saturation pulses in a light adapted environment. Saturation flash frequency may be set differently for dark quenching relaxation work.

Saturation pulse frequency

By using the Stepped Actinic Test in the Kinetic Protocol, one can check to see if there is enough time between pulses to allow complete relaxation of saturation pulse NPQ. If not, then saturation pulse related NPQ would cause an error. Rosenqvist and van Kooten (2006) state that a time of between 1 and 2 minutes is required for complete relaxation of saturation pulse NPQ. When in doubt use two minutes. (We have found that more time than 2 minutes may be required between saturation flashes in the dark for longer measurements).

If one wants to test for the minimum time required to eliminate saturation pulse NPQ, then using the quenching test, with different times between saturation pulses, will provide the answer. The test should allow about twenty minutes for the plant to reach steady state photosynthesis (Maxwell and Johnson 2000) or 20 to 35 minutes at high light levels. If the F_M' values continue to decline and the F' values continue to change, then the pulses are too close together. If you are looking for a safe time to use for duration between saturation flashes, 2 minutes is suggested as the shortest safe time by Rosenqvist and van Kooten (2006). For quenching relaxation measurements of q_M longer times between saturation pulses may be required from our experience for longer relaxation times.

Saturation pulse duration

On the OS5p+, the saturation pulse duration is set at the default value of 0.8 seconds. This is generally ideal for higher land plants, however, times of 0.3 to 2 seconds have been used Rosenqvist and van Kooten (2006). The ideal saturation duration is determined as the value that completely saturates PSII without causing saturation pulse curvature at the trailing edge of the saturation pulse. This is a form of saturation pulse NPQ, and it lowers the average value of F_M, or F_M' used in F_V/F_M quenching, and Y(II) measurements. The saturation pulse duration can be set on the OS5p+ from 0.40 seconds to 2.00 seconds. The OS5p+ uses a rolling eight point average with a duration of 25ms to determine the highest F_M or F_M' value. This prevents saturation flash NPQ from causing an error even if the saturation flash duration is set to be too long. This ensures that reliable measurements are made for land plants, algae, or cyanobacteria. With modulated fluorometry, the value measured at F_M, or F_M' represents multiple turn-overs of Q_A According to Schreiber (1995) the ideal saturation pulse duration for algae and cyanobacteria range from 25ms to 50ms.

Length of quenching test.

The leaf must be at steady state photosynthesis before most quenching measurements are made. This means that the leaf is exposed to a specific light level for between twenty and thirty five minutes. When in doubt, use thirty five minutes at a specific light intensity level. Use the PAR Clip with a tripod and a dark shroud, the PAR clip and a tripod for pre-dawn measurements, or the PAR clip and a tripod in a darkened room. The actinic light level can be measured if a PAR Clip is used, and the intensity is held stable. If measuring qI, one may want to expose samples to non- photoinhibitory light conditions during the day for three days. This should eliminate photoinhibition. To measure the effects of chronic photoinhibition the sample must then be dark adapted and then the routine can be run. F_M can be measured without photoinhibition. It is then exposed to photoinhibitory conditions for several hours. After this exposure, the quenching relaxation test can be run. Times for q_E, and q_M should be selected and tested. q_I is what is left after measuring q_M.

For other relaxation parameters, it is common to dark adapt over night and light adapt to steady state photosynthesis. According to Cazzaniga (2013) this can take up to 35 minutes. While there will be some residual qI in field plants, it should not matter as long as the light history is similar between samples.

Dark Adaptation – How long is long enough?

Dark adaptation is a technique used in some chlorophyll fluorescence measurements to fix a non-stressed reference point relative to various measurements (Maxwell and Johnson 2000). Deciding where to put that reference is based on an understanding of plant mechanisms that can affect measurements, and what one wants to measure.

With the latest information regarding chloroplast migration it may be prudent to dark adapt for at least 35 minutes. Dark adaption times of ten minutes, twenty minutes, thirty five minutes, forty minutes and sixty minutes have been used for terrestrial plants, and some researchers use pre-dawn values. The choice is dependant on whether one is measuring plant stress, or measuring plant mechanisms in different species and under different growing conditions.

To obtain reliable modulated F_V/F_M values, decisions need to be made for control and test measurements. The plant mechanisms listed below will lower F_M, and possibly raise F_O, changing F_V/F_M measurements downward like other types of plant stress. One must decide which mechanisms are of concern and dark adapt accordingly.

F_V/F_M is affected by both photochemical and non-photochemical factors. If a leaf is dark adapted and measured, then subjected to very high light levels for hours, then dark adapted again and re-measured, the first measurement will be higher then the second measurement. The decline in F_V/F_M measurement may be due to a decrease in reaction centers capable of photochemistry or un-reversed non-photochemical quenching. (Baker N.R., Oxborough K. 2004)

Papageorgiou reports that results may vary greatly depending on how long dark adaptation is done. A few minutes of dark adaptation is enough to re-oxidize the plastoquinone pool and the CaMn4OxCly cluster, while longer periods deplete respiratory substrates through respiration in cyanobacteria and chlororespiration in higher plants and algae. Longer times will also deplete ATP pools, and trans-membrane ion concentration gradients. Dark adaptation also shifts higher plants and algae toward state 1 conditions and cyanobacteria to state 2 conditions. (Papageorgiou G.C. Tismmilli-Michael M. Stamatakis K. 2007) There is some evidence the fluorescence change attributed to state transitions may in fact be due to chloroplast migrations around the cell vacuole.

Rapid acting photo-protective mechanisms activated by exposure to variable light intensities (designated in the parameters q_E and Y(NPQ) are the xanthophyll cycle and thylakoid lumen ph. They relax in a few minutes during dark adaptation. (Muller, Niyogi 2001),(Kramer D. M., Johnson G., Kiirats O., Edwards G. (2004). According to Lichtenthaler (1999) this time is 4-6 minutes. According to Baker (2008) this time can be longer in the field. He reports times up to 7 minutes.

State I – State 2 transition quenching (called q_T) is most significant at lower light levels in terrestrial plants and can represent more than 60% of quenching at low light levels. At high light levels it represents about 6% of total quenching. State transition quenching relaxes in 15 to 20

minutes in terrestrial plants. (Lichtenthaler H. Burkart S 1999) (Again, this may be caused by chloroplast migration.)

Recently it was discovered that the fluorescence change that was thought to be caused by state transitions is caused by chloroplast migration at least at high actinic light levels, and at least in dicots. (Cazzaniga 2013) It may be the source of chlorophyll fluorescence in Monocots as well because chloroplast migration exists in monocots as well (Maai 2011). In addition, it was thought that the fluorescence change from 20 to 30 minutes was due to the effects of acute photo-inhibition caused by exposure to high light intensities. Relaxation could also be reversed with 20 to 30 minutes of dark adaption (Theile, Krause & Winter 1998). With Cazzaniga's research acute photoinhibition is likely chloroplast migration (Cazzaniga 2013). It can take 20 to 35 minutes for chloroplast migration to be completed.

The reversal of chronic photo-inhibition, caused by several hours of high light exposure, starts to relax at about 40 minutes and may take 30 to 60 hours to fully relax under dark adaptation (Lichtenthaler H. & Babani F. (2004) (Theile, Krause & Winter 1998)

When making longer quenching and quenching relaxation parameter measurements related to photo-inhibition and photo damage mechanisms that are common in chronic high light stress, high heat stress, cold stress and over wintering stress, one should understand that it could take days for full relaxation or repair of the non-photochemical quenching parameters, q_I and Y(NO), to pre-stress conditions. To get an accurate control value for F_M and F_O under chronic photo-inhibition conditions (components of non-photochemical quenching parameters) it is common to dark adapt for a full night, or 24 hours. (Maxwell and Johnson 2000) In some cases longer times may be appropriate. Lichtenthaler (2004) Even so It is expected that there is some residual un-relaxed NPQ built into the measurement. To measure q_I a few days under shaded conditions may be required for full relaxation of q_I. To measure photoinhibition more accurately it may be best to partially shade samples for a few day to eliminate residual photoinhibition.

In Aquatic Plants Gorbunov (2001) is a good source for corals, and Consalvey (2004) is a good source for Algae. For information regarding dark adaption for rapid light curves Rascher 2000 is a good source. Ralph (2004) describes momentary dark adaptation for Rapid Light Curves.

The use of far red pre-illumination that is available on some fluorometers is designed to rapidly re-oxidize PSII by activating PSI. This can be valuable in field work, (Maxwell and Johnson 2000), but it does not affect the relaxation of non-photo-chemical quenching mechanisms. Consalvey (2004).

Dark adaptation can be accomplished by using dark adaptation leaf clips or cuvettes. Some researchers use hundreds of inexpensive clips to make measurements on larger populations quickly. Shrouds or darkened rooms may also be used. As stated before, some researchers use pre-dawn measurements.

In review, it is important to take a few things into account. Reliable dark adaptation times can vary by species, plant light history, the fluorescence parameter of interest, and the type of stress that needs to be measured. When dealing with a new species or an unknown photo-history it is

best to test for maximum and stable F_V/F_M at different dark adapted times for best results. When testing for optimal dark adapting times it is important to use samples that have been exposed to the maximum light conditions that will occur during the experiment.

Cookbook checklist before making NPQ and other quenching measurements.

Quenching measurement parameters, such as NPQ, are the least understood, and most often misused parameters that are available with advanced chlorophyll fluorometers.

This application note is designed to improve the understanding of proper quenching protocol usage.

While the puddle model, that based on a one antennae per reaction center model, is no longer credible, puddle model based parameters continue to be used. The newer model, that is based on shared antennae, is the lake model. The Kramer lake model (Kramer 2004) uses F_O' or quenched F_O in most of its measuring parameters. In addition, it provided q_L or a photochemical parameter to replace q_P, a puddle model parameter. The Hendrickson lake model offers simplified Kramer equations without F_O' (Hendrickson 2004). The Hendrickson lake model equations do not have an equivalent to qL or photochemical quenching; however, Klughammer shows that NPQ can be resurrected from the puddle model to the lake model using Hendrickson's equations, NPQ= Y(NPQ)/Y(NO). Furthermore, both sets of equations provide Y(II), Y(NPQ), and Y(NO). For an in-depth discussion of the differences of each set of equations see Quenching application note available for free at www.optisci.com .

To get reliable measurements, one should follow tested guidelines.

1. Dark-adapt properly knowing the plant's light history. It takes only a few minutes for the xanthophyll cycle and the ΔpH of the thylakoid lumen to return to a dark-adapted state. State transitions, however, take between fifteen to twenty minutes. These times can vary somewhat in field plants and can take slightly longer (Baker 2004). Recently, it was found that the fluorescence change that was thought to be due to state transitions and acute photoinhibition was really caused by chloroplast migrations (Cazzaniga 2013). q_M, the fluorescence intensity value change primarily by chloroplast migration, can represent about 30% of NPQ at high actinic light levels. Furthermore, it takes from 20 to 35 minutes for chloroplast migration to completely adjust in the light, and relax in the dark.

In addition, field plants and other plants that have been exposed to high light photoinhibition conditions for a number of hours, will retain a certain amount of NPQ or chronic photoinhibition for up to 30 to 60 hours (Lichtenthaler 2004). This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into summer field measurements of F_V/F_M, and other displayed quenching parameters. For this reason, it is important to only compare samples with a similar light history. When doing quenching measurements on field

plants, it is common for researchers to choose pre-dawn dark adaptation values. It is not common to use shorter times to study quenching. (For more information, see dark adaptation application note.) If one is studying photoinhibition, it is a good idea to partially shade samples for a few days, if possible, to ensure that all photoinhibition has relaxed or repaired to get a base line.

2. Samples that are compared, must have the same F_V/F_M values. Quenching measurements of different samples with different F_V/F_M values should not be compared (Baker 2008). F_V/F_M is used as the measuring standard for non-photochemical quenching measurements, and if the measuring standard is different, the quenching values are meaningless. Comparing values from samples with different F_V/F_M values is like measuring items with a ruler that has dimensions that change.

3. Modulation light intensity setting (F_V/F_M is $(F_M-F_O)/F_M$). F_O, or minimum fluorescence is a dark-adapted value made by exposing the leaf antennae to a very low intensity modulated measuring light, that allows measurement, but not high enough to photochemically reduce Q_A in the sample. The modulated light may be set manually or it may be set using the "Auto Setup" button under "Change Settings" in the quenching protocol. The automated modulated light intensity setting allows easy adjustment.

4. Leaves must be at steady state photosynthesis for reliable measurement. It was previously thought that for most quenching measurements, that it took between 15 and 20 minutes at a new light level (Maxwell and Johnson 2000) to reach steady state. However, recent research shows that chloroplast migration takes between 20 minutes and 35 minutes to fully adjust. There is strong evidence that chloroplast migration can be responsible for up to 30% of NPQ at high actinic light levels in dicots (Cazzaniga 2013) and probably moncots as well (Maai 2011). The fluorescence change thought to be due to state transitions and acute photoinhibition is most likely due to chloroplast migration. For this reason, longer pre-illumination times are likely to be required for reliable measurement. For example, if there are 18 saturation pulses spaced 120 seconds apart, the leaf will be exposed to the actinic light for 36 minutes after dark adaptation. Since an internal fluorometer artificial light source is normally used, the test allows one to compare leaves as long as the F_V/F_M values are the same. According to Klughammer (2008), the only non-photochemical parameter that does not have to be taken at steady state photosynthesis is Y(NO) from Hendrickson.

5. Use a fluorometer with a stable actinic light output. Depending on the brand and type of fluorometer, the intensity output of the actinic light can change over time. When an actinic light is on, it can heat the fluorometer and cause a lowering of the light output. The intensity of the actinic LED light source output changes as the heat from the lamp changes the lamp temperature. The OS5p+ uses the PAR Clip to measure actinic light output, and adjust the intensity of the light source as necessary to maintain a constant light level to +- 3 μmols over long periods of time. (Use the PAR clip on a tripod. A dark shroud, pre-dawn measurement, or a darkened room may be used for measurement). Changes in actinic light intensity during measurement can be the source of significant error.

6. Shade leaves vs. Sun leaves. – The Y(II) ratio will be higher on Sun leaves than on shade leaves (Lichtenthaler 2004).

7. Leaf orientation is not important because an artificial actinic light source is used.

8. It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants (Reuter and Robinson 1997). Other sampling plans are also used.

9. The duration of the saturation pulse should be between 0.5 seconds and 1.5 seconds for higher plants (Rosenqvist and van Kooten 2006), and 25 to 50 milliseconds for Phytoplankton and cyanobacteria (Schreiber 2005) . Times outside these ranges increase the error in Y(II) and quenching measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity. Longer durations can create a form of saturation pulse NPQ that rounds the tail end of the saturation pulse maximum value, and can reduce the average maximum saturation pulse value. (Rosenqvist and van Kooten 2006). The OS1p and OS5p+ use a special algorithm to prevent errors caused by saturation pulse NPQ. A rolling 25 ms., 8 point average of the highest F_M and F_M' values is used to prevent this error. This ensures that the correct value is reported as long as the saturation pulse width is wide enough.

10. Saturation pulse intensity. Saturation pulse intensity is more of an issue with the light adapted Y(II) than with dark adapted F_V/F_M . While shade leaves will saturate at a few hundred µmols, sun leaves will usually saturate below 1,500 µmols (Ralph 2005). A very intense saturation flash intensity does not damage light adapted samples, but may damage dark-adapted samples under cold stress conditions, if the saturation flash happens too frequently in the dark. Multiflash or square saturation flashes may be selected and used. See Multiflash for more details. For a single test, it is unlikely that intense flashes in the dark are a problem; however, for extended periods, the frequency of saturation flashes should be considered. It is recommended that maximum intensity should be used for all quenching measurements. Saturation pulses used at night for extended tests, should be at least ten minutes apart to one hour apart to prevent photo-damage from saturation pulses (Porcar-Castell A. 2008). The OS5p+ offers single relaxation saturation flash measurements at specifically selected times for q_E and other parameters, or a series of general relaxation saturation flashes spaced at a programmable distance apart. Both schemes can be used together, but saturation flashes should be at least two minute apart to prevent a build up of saturation pulse NPQ.

11. The time between saturation pulses is important. Rosenqvist and van Kooten (2006) state that between one to two minutes is required for complete relaxation of saturation pulse NPQ. If saturation pulses are not separated by this distance range, then an error caused by this type of saturation pulse NPQ will result. It will accumulate with each saturation pulse. When in doubt, space saturation pulses 120 seconds apart or more.

12. Overlap of PSI fluorescence -Part of the minimum fluorescence, the F_O parameter, in F_V/F_M ($(F_M - F_O)/F_M$), contains PSI fluorescence as well as PSII fluorescence. With F_V/F_M, one is trying to measure the maximum variable fluorescence of PSII in a dark-adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces a small non-variable error. In C_3 plants, about 30% of F_O fluorescence is due to PSI, and in C_4 plants about 50% of F_O fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in F_M in C_3 plants, and about 12% in C_4 plants. (Pfundle 1998). This not a problem when comparing quenching measurements for plant stress because, PSI fluorescence does not change with light level or plant stress.

13. Leaf fluorescence heterogeneity, presents itself as patchy fluorescence on measurements of different parts of the leaf. It has been found to occur under cold stress conditions, with biotic stress, and under drought stress conditions. By using multiple measurements on the same leaf a

sampling plan and averaging, heterogeneity can be over come (Buschmann C. in correspondence by e-mail 2008).

14. One can set the q_E and q_M (or q_T & q_Z) values to any minute value, but there could then be additional saturation flashes between the general saturation flashes and therefore saturation flash NPQ could create an error if they are less than 120 seconds apart. For example: If general quenching relaxation saturation flashes are 2 minute apart, q_E might be set at 4 minutes and q_M at 36 minutes. q_E and the intermediate value may also be set without measuring general relaxation saturation flashes, at any minute time. It is believed that there is some overlap between q_E and intermediate relaxation parameters. q_E can be set from 1 minute to 10 minutes. Baker (2008) states that it is shorter for non-field plants and longer for field plants. He states that times up to 7 minutes are possible in field plants. q_M or chloroplast migration takes between 20 to 35 minutes (Cazzaniga 2013), q_T or state transitions range from 15 minutes to 20 minutes (Ruban 2009). q_Z or an unknown longer NPQ mechanism ranges from 20 minutes to 30 minutes (Nilkens 2010).

15. Far red light - The built in actinic light has been filtered to eliminate far red light that excites PSI. This allows more control. One can select the use of far red light during any and all phases of the quenching protocol as desired. Of course, sun light contains far red light. Far red light may be turned on or off for various parts of quenching tests, or can be turned on or off for the entire quenching protocol. It has been used to pre-illuminate dark-adapted leaves with far-red light. When this feature is used for five to ten seconds before an F_V/F_M measurement takes place, it activates PSI, and ensures that all electrons have been drained from PSII before the measurement of F_O. While this feature ensures that PSII is completely re-oxidized, it does not relax the xanthophyll cycle, state transitions, chloroplast migrations, or photoinhibition. Time is still required in a darkened environment to relax all forms of NPQ and to obtain reliable quenching values. Far red light is used after saturation flashes to measure F_O' a parameter used in many Kramer lake model parameters, and in many puddle model parameters.

The best experiments are ones that take these issues into account. PSI fluorescence is involved in all measurements. It does not vary with light level or plant stress (Schreiber 2004). With this in mind, comparing samples with similar light histories allows comparison of many types of plant stress.

The Plant Stress Guide provided by Opti-Sciences www.optisci.com , references papers that deal with specific types of plant stress and limitations of different chlorophyll fluorescence parameters for measuring plant stress.

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Chapter 2 • The OS5p+ Hardware

Introduction

The OS5p+ Modulated Fluorometer is a multipurpose portable measuring instrument designed to precisely measure chlorophyll fluorescence. The system is simple to use, light in weight, and battery powered, making it an excellent choice for field studies. It represents culmination of several years of testing and development and was engineered to give many years of reliable service. This chapter provides general information about the OS5p+ Modulated Fluorometer.

Key Features

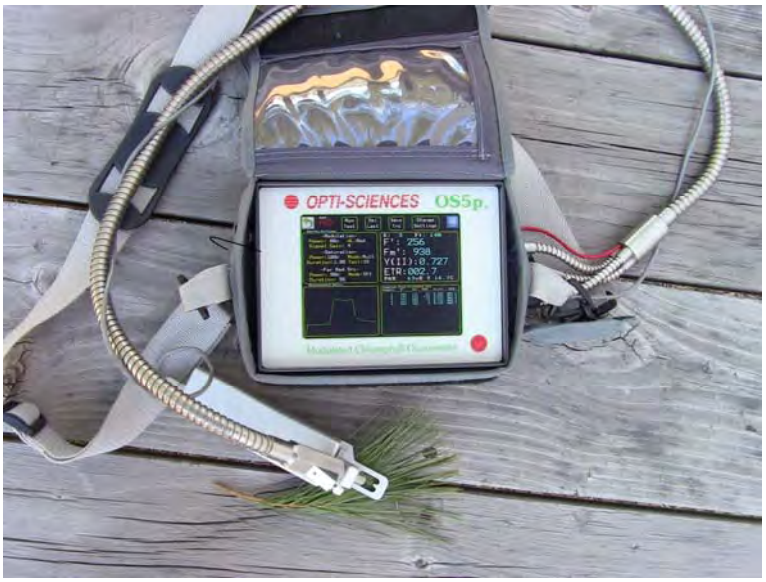
The OS5p+ includes the following features:

- A PAR Clip to measure PAR, leaf temperature, and ETR.
- A *stable actinic light source* that may be used to maintain a stable light intensity for long periods of time. Ideal for quenching measurements or pre-illumination of samples.
- *Multi-Flash* option is included – F_M' correction protocol option for light adapted and quenching tests. A multiple phased single saturation flash is used. The protocol originally based on Loriaux 2006, has been updated to reflect Loriaux 2013 research. This corrects for the F_M' measuring error found at high actinic light levels.
- Quenching protocols included: *Kramer lake model*, *Hendrickson Lake model with NPQ* resurrected to the lake model by Klughammer, *puddle model*, and *quenching relaxation* measurement.
- Red and blue modulated measuring lights.
- Advanced *Strasser OJIP* plant stress measuring protocol with graphic overlay. All of Strasser's parameters are reported in the data file and the most used parameters including PI_{ABS} are reported on the measuring screen.
- *Vredenberg OJIP quenching protocol.* This is special protocol developed with Vredenberg to investigate Non-photochemical quenching during the OJIP fluorescence rise with variable time resolution.
- High resolution color graphic touch screen user interface.
- Fast test protocols for dark adapted F_V/F_M, or F_V/F_O, and light adapted Y(II) and ETR.
- An automated modulated light set up protocol to save time and prevent errors. Of course it may also be set up manually as well.
- Far red light can be used to stimulate PSI, reoxidize PSII, or measure Fo'.
- USB, MMC/SD data card outputs
- Light weight design excellent for field studies.
- Fully portable Nickel Metal Hydride battery operation. It can also be operated from a AC outlet. A Charge will last up to ten hours.
- 2 Gbyte of non volatile flash memory
- Versatile software, optimized for variations in testing procedures.
- *Rapid light Curves* are included as standard with *onboard Eilers and Peters curve fitting* software include. Multiple curves may be overlaid on the measuring screen.
- It comes with a durable transport and storage case.
- Options Include:
    1. Extended life battery belt for remote locations and extreme hot and cold environments
    2. Tripods
    3. Additional Dark Clips
    4. Algae cuvette

Physical Features -Top Panel

Top Panel:

Figure 2-1 • Front Panel of OS5p+



Front Panel of OS5p+

Touch screen showing the Y(II) measuring protocol using F_M' corrections on the screen.

The Red round button is for OFF and ON

Left Side Panel:

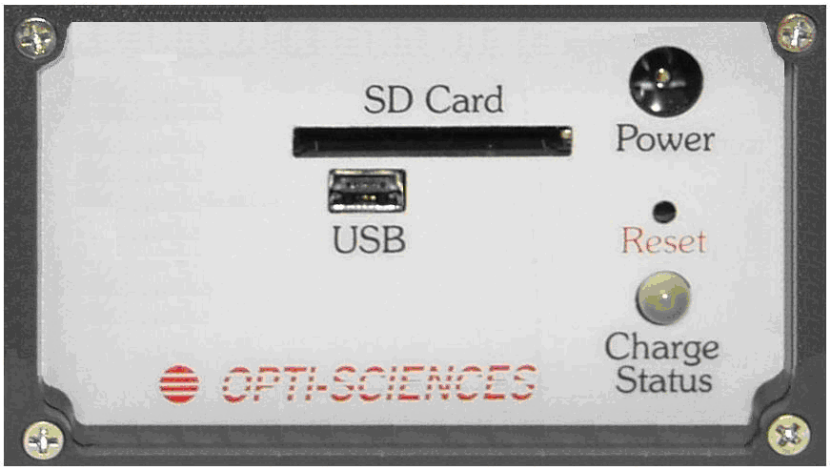


Figure 2-2 • OS5p+ left side panel

SD Card Slot – used for MMC/SD format data cards up to two GByte. The port can be used for data transfer to other computers or it allows multiple users to keep their measuring setups and data separate from other users.

USB port –used for data output to computer. Files are comma delineated for direct opening into Excel, Mat Lab, or other spread sheets.

Power – used for the battery charger. The charger also doubles as a mains connector. The Charge status light is green when the unit is fully charged. It is red when it is charging and not fully charged. The OS5p+ only charges when the unit is turned off.

Reset hole – By inserting a pin or paper clip, the system can be rebooted if the system should lock up.

Charge Status Light – The light turns green when the Nickel Metal Hydride battery is fully charged. It will be red when it is not. It only turns on when the OS5p+ is turned off and the unit is plugged in.

Right Side Panel:

Figure 2-3 • OS5p+ right side panel

Accessory port – connection for PAR clip.

Fiber-optic light guide connectors – male connectors for the tree randomized fiber optic bundle connectors. Any of the fiber optic female connectors may be attached to any of the right side panel connectors.

Remote Connector – This is used for the trigger switch wire connector attached to the fiber optic bundle, and it is used to trigger measurements from a button on the other end of the fiber optic bundle.

Picture of PAR Clip on optional articulating arm stand

This picture shows the correct placement of the fiber optic bundle on the PAR Clip. The fiber bundle should be moved in as close as possible to the leaf. PAR Clips are highly recommended for field light adapted use. Y(II) varies not only with plant stress but also with light level. Only samples at the same light level and with the same light history should be compared. Sun leaves respond differently to light levels than shade leaves.

Hardware Overview and Measurement Principles

The OS5p+ is a portable pulse modulated chlorophyll fluorescence fluorometer It is completely self-contained, eliminating the need for any external hardware or computing device (palmtop computer, etc.). All detection and data logging components are on-board.

Fluorescence is measured by a modulated light sources in the OS5p+. The standard and more commonly used red modulated source is a diode that peaks at 660 nm with filters blocking radiation longer than 690 nm. The average intensity of this modulated light is adjustable from 0 to 1 $\mu mol/m^2/s$. It is adjusted to samples so that it does not drive photo-chemical reduction of Q_A in dark adapted tests but is high enough to make measurements. The source output is optically monitored inside its assembly to correct for variations in output due to changes in ambient temperature. The optional, less commonly used, blue modulated diode peaks at 450nm and can be used for algae work or for work with higher plants as well. Detection is done in the 700 to 750 nm range using a sensitive PIN silicon photodiode with appropriate filtering to narrow the optical response. A low-noise preamplifier is mounted in the detector assembly as well. The detection method is a modified lock-in amplifier design which allows the use of a very low average modulated light energy. It is the modulated light source that allows actual measurement of F_o, and F_o' as well at the measurement of light adapted quantum yield of PSII. The gain control is adjustable for optimum signal to noise ratio. The capture rate is automatically set for the test that is selected. An added benefit of this system is its ability to disregard extraneous ambient light. This instruments has an automated modulation light adjustment routine the sets the correct intensity and gain for the modulated light. Of course it can still be set manually.

Light sources

There are two standard measuring light sources present in the OS5p+:

The Modulation Measuring Light Sources

Modulated light source This is the light source that makes light adapted quantum photosynthetic yield measurements possible along with direct measurements of F_o and F_o' The modulated light source is used at an intensity range that is too low to drive photosynthesis and yet allows fluorescence measurement of pre-photosynthesis Fo, and post photosynthesis Fo' This light source is turned off and on at a particular frequency. Opti-Sciences adjusts the frequency automatically for optimal use. In addition, this instruments has an automated modulation light adjustment routine that sets the correct intensity and gain for the modulated light. Of course it can still be set manually.

1. The first modulation source has a 660nm output used exclusively for measuring of the fluorescence signal. It's intensity may be varied to adjust for different species and conditions in dark adapted samples and for use with the PAR clip on light and dark adapted samples.
2. The second modulation source is a 450nm LED and is also used exclusively for measuring of the fluorescence signal. It's intensity may be varied to adjust for dark clips, Par Clips, and sample differences as well. While this source can be used for standard fluorescence measurements, it can also be used for green algae, diatoms, and dinoflagellates. It should also be use when cyanobacteria are present in the sample, and one wants to exclude results of cyanobacteria. Because of absorption differences, using the blue modulated light source will change ETR values. In the equation used for blue modulated light, leaf absorption should be changed to 0.94, on average, from 0.84. for best correlation to red modulated light values. This can be done in Excel using the measuring file. ETR= Yield X PAR X 0.94 X 0.5 (for blue modulated light).

Saturation Light Source is a white light LED with a maximum intensity of 15,000 μmols.

Saturation pulse is a short pulse of intense light designed to fully reduce a leaf's PSII system. For higher plants, the optimal duration of the saturation pulse is between 0.5 seconds and 1.5 second (Rosenqvist and van Kooten 2006). For Algae and cyanobacteria, the duration must be shorter to provide accurate measurements 25 ms to 50 ms. (Schreiber 1999). Opti-Sciences uses 0.8 seconds as the default value for land plants however the duration can be set between 0.4 seconds and 2.0 seconds by pressing the Y(II) graph on the Y(II) measuring screen and pressing the duration button. Opti-Sciences uses an 8 point 25 ms rolling detection average to determine the highest F_M, and F_M' independently of saturation pulse duration. This ensures that as long as the duration is long enough, the optimal measurement will be made. The Saturation pulse light source is a high intensity white light LED. It is 15,000 μmols at 100% intensity when used with a dark clip, and about 7,500 μmols when used with the PAR Clip at 100% intensity.

Another way to measure F_M' and Y(II) under high actinic light conditions is a standard option called Multiflash. This instrument incorporates the latest research and most accepted method for F_M' correction. Multi-Flash a multiple phased single saturation flash may be used to correct for F_M' and ETR values in samples that have been exposed to high actinic light The method used follows the research by Loriaux 2013. See Multi-flash for more details.

The LED Actinic Light Source

An actinic source is a light source that drives photosynthesis. It may be the sun or an artificial light source. Using an artificial light source has the advantage that it can be set to a specific intensity, eliminate the variability of sun light shading, and partly cloud day cover. The OS5p+ has a high intensity white light LED, with an intense blue spectrum that is used as an actinic light source to provide more natural lighting conditions. When used in conjunction with the PAR clip, the PAR light intensity at the leaf can be set and maintained at a constant level for long periods of time. This eliminates the error caused by heat. If the light source was not stabilized, the intensity of the actinic light source would continue to drop as the lamp and the chlorophyll fluorometer heat up. This is why some manufactures recommend the use of external actinic light sources for quenching measurements, and sample pre-illumination.

It was recently found that white actinic light sources or intense blue actinic light sources are necessary for chloroplast migration to occur as it does in nature (Cazzaniga 2013). An intense red light will not significantly affect chloroplast migration. According to Cazzaniga (2013), q_M, or chloroplast migration can represent up to 30% of NPQ at high actinic light levels. As a result it is important to have a white light source with an intense blue spectrum.

The Far Red Source

The peak wavelength for the Far Red source is ~735nm. Far red light is a light source that drives PSI without driving PSII. This has the effect of draining the remaining electrons from PSII quickly to provide an optimal oxidized state in PSII. It is used for determination of F_o'. Light at these wavelengths allows electron transfer from PSII to PSI, and it allows the fast re-oxidation of PSII. The far red source may be used in the F_V/F_M protocol to rapidly re-oxidize PSII before measurement, or it may be used all of the time.

During the quantum yield of PSII test when artificial internal illumination is used, far red light is filtered out of both internal illuminators. Therefore, to closely replicate solar radiation that has both PAR radiation and far-red radiation to drive both PSII and PSI, the far red light should be on during internal illuminator actinic illumination. Both intensity and duration are adjustable.

The Fiber Optic Light Guide

The optical signals are transferred to and from the sample by a custom-designed trifurcated fiber optic light guide, with randomly mixed fibers. One end of this fiber bundle has three BNC (twist-lock) connectors for the three BNC male connectors on the OS5p+ right side panel. Any fiber optic BNC connector can be attached to any OS5p+ BNC connector. In addition there is a trigger switch on the far end of the bundle from the BNC Connectors. This switch can be used to take a measurement, or one can press the enter button on the OS5p+ front panel, The trigger switch is connected electronically by a wire running down the fiber optic cable, and attaches to the

"remote" port on the right side panel. The other end provides a comfortable fiber bundle grip and an interface section designed to fit into the many cuvette options.

Electronics

The system has 32 bit microprocessor with two Gigabytes of flash memory.
No data will be lost due to loss of power.

Storage and Carrying Case

The OS5p+ is housed in a durable wear resistant ABS plastic case. This is the same material that is used in the manufacture of motorcycle helmets. It has been designed to be used as carry on luggage on airplanes. It also comes with a nylon field bag with a pocket to carry accessories, and a shoulder strap for ergonomic field use.

Chapter 3 • Operating the OS5p+

Introduction

The OS5p+ is shipped with the battery connected. It is a good idea to give the battery a full overnight charge before the first use.

Connect the three fiber optic light guide BNC ends to any of the BNC ports on the right side panel of OS5p+. Make sure that all three of the BNC connectors are locked in place. The remote trigger switch (the 1/16" red wire on the fiber) connects to the jack labeled “remote” on the right side panel.

Introduction to running tests

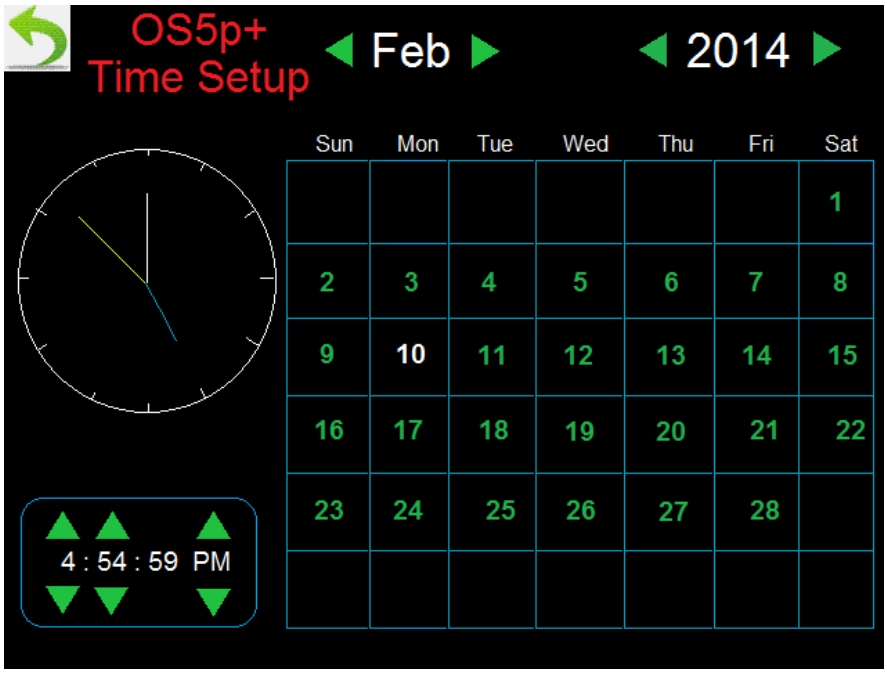
As with any electronic measuring tool, it is important to turn on the tool before use and let the electronic temperatures reach operating range. This takes about 10 minutes for best results.

Press the red main power button on the front panel. Then the main menu screen shown below appears.

Main Menu

Main Menu & Clock

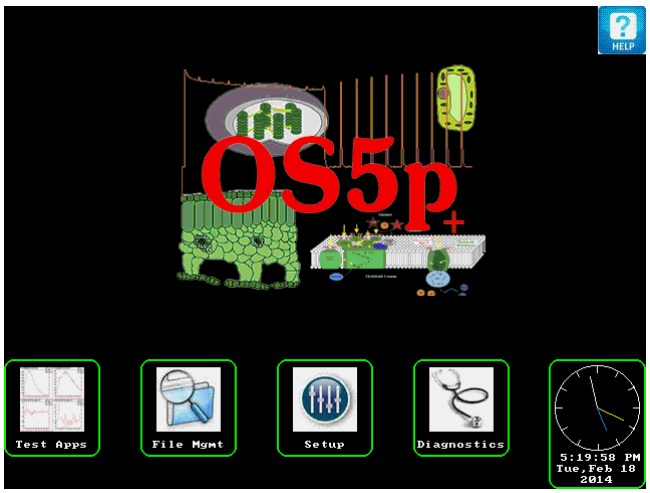
Press the touch screen on the “Clock” icon and a second screen seen below will appear.



The default time is set for US eastern standard time (12 hour format).

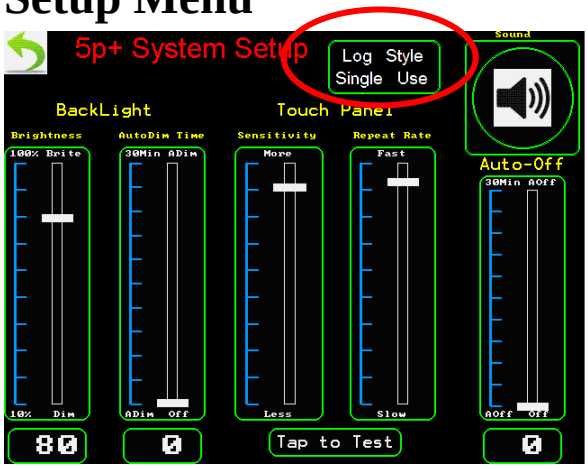
With this screen one can adjust the time and date. Press the triangle buttons to change the values, and touch the day to select the date.

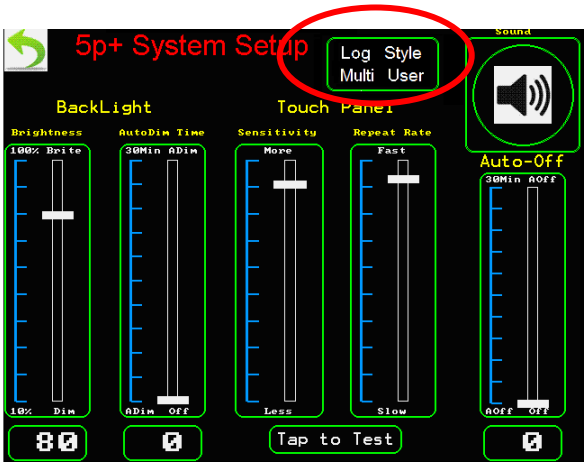
Touch the arrow in the upper left hand corner to go back to the main screen. All changes will be saved by pressing the return arrow in the upper left corner.



Touch the setup icon next.

Setup Menu





This set up screen will appear.

Important!!!

The Log Style button The two choices here are for **Multi User** and for **Single Use** options. The default method is the Single Use option. In the Single Use Option, data files are stored to the internal flash drive and separate **Preset** files (parameter adjustments) are stored in a Presets file area stored separately on the flash drive. They may also be transferred to and from the removable data card, recalled for use in the active memory or transferred by USB port.

Important!!!

The Multi User Option prevents the internal flash drive from being used for data storage and for Presets file storage. Instead, all Data files, and Preset files are only stored to removable data cards for use. This way, the fluorometer may be used by multiple researchers. Each researcher keeps their own data and Preset files on their own data card. In this mode, the

OS5p+ will prompt the user to insert their data card, the OS5p+ will read the name on the card and display it on the screen. This is done to verify the card that has been inserted. That way, research can be kept separately and kept safely. When a data card is inserted into the instrument for the first time, prompting will require naming of the card using the internal digital keyboard. From then on, all data and Preset files are recorded and can only be retrieved using the data card. Don't lose your data card!

The **Speaker button** allows an audible beep when measurements are made and provides other audible indications as well. This may be turned off or on by pressing the button with the speaker.

The Auto-Off button allows the instrument to turn off automatically when not in use. The time is adjustable between zero minutes and twenty minutes by using the + and – buttons. When set at zero, this feature is turned off.

Touch panel – The screen touch sensitivity and repeater rate can be adjusted to personal preferences. The Tap test is designed to test your settings.

The Auto-Dim - is a battery saving feature. It will dim the screen at the time selected. If it is set to zero it is turned off.

The brightness - may be set to personal preference. It can be set to the brightest setting for field use and to a lower setting for lab use. It is designed as a battery charge saving device.

Diagnostics

This series of screens is used to diagnose problems or to reset the instrument to default settings. This section can be used to provide battery charge information, remaining memory capacity, or test various components for reliable operation.

Opening the OS5p+ fluorometer case voids the warranty!!!!!!!!!!!!!!!

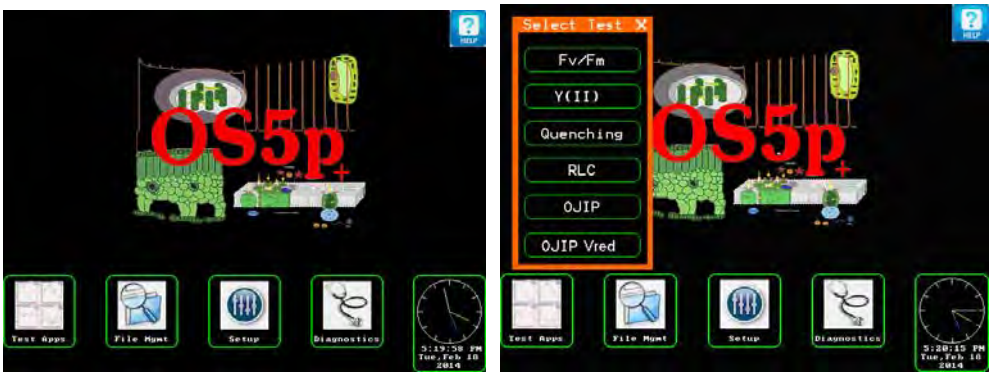
The pictures above are a map of the diagnostic screens. The main screen, above left, appears when “Diagnostics” is tapped on the main OS5p+ screen. By tapping green on the main diagnostic screen page, windows the other screens appear as shown. Use the green return arrow in the upper left hand corner to return to the previous page. The TPanel Test can be used to see how well the touch panel is calibrated. Dots and lines should appear where touch occurs. If not, contact Opti-Sciences.

“Factory Reset” – Tapping this button will reset all active memory values to the original default factory settings. It will not erase Preset files, data files, or Script files.

Fluorometer Diagnostics can be viewed by tapping inside the green window under **Fluorometer System**. It allows display of various light source signals to test if the various light sources and sensors are working properly. If they are not, contact Opti-Sciences with the information.

Eternal Sensors - Tap the green window and the screen on the upper right hand will appear. At this time it is for testing the PAR Clip measurement of PAR and leaf temperature. If there are problems, contact Opti-Sciences.

System Power Usage – Tap the green window and the screen on the lower right hand side will appear. It will allow viewing of the various power values. The battery capacity will tell you how much charge is left. Other meters can be valuable for diagnosing problems. Contact Opti-Sciences if you encounter a problem. The Charge Graph must be pressed twice for viewing. The second time will be several seconds after the first time. The system will stop charging after it is fully charged. If the power cord is removed and plugged in again, the charging cycle will start again.



Test Apps

The OS5p+ has different test apps or protocols, each protocol providing the necessary setup and data logging for each type of test.

Press "Test Apps" using the touch screen. Next select the protocol of interest by touching the a green window inside the orange window. To go back touch the "X" in the upper right corner of the orange window. The list below includes the test protocols:

1. Fv/Fm is the F_V/F_M Protocol — Dark adapted test for maximum quantum yield.
2. Y(II) – Quantum Yield of PSII Protocol — Light adapted test of (PSII) for effective quantum photochemical yield - Y(II) or ΔF/Fm' and ETR or electron transport rate.
3. Quenching - There are several different protocol options in this category. One chooses the protocol and option of interest. They include:
    - Kramer lake model quenching parameters, including Y(II), Y(NPQ), Y(NO), and qL at steady state photosynthesis. Kramer uses F_O' in his parameters.
    - Hendrickson lake model quenching parameters with NPQ resurrected from the puddle model by Klughammer. It includes parameters NPQ, Y(II), Y(NPQ), Y(NO), PAR and leaf temperature with the PAR Clip accessory. If the customer does not select a protocol for quenching this option is supplied as the default protocol. It offers simplified lake model quenching parameters with the addition of NPQ, resurrected from the puddle model. Hendrickson uses F_O instead of F_O' in his parameters.
    - Puddle model quenching parameters include: Y(II), NPQ, q_N, q_P. q_N and q_P use F_O' in their formulas while Y(II) and NPQ do not.
    - Quenching relaxation test can be used with the various protocols, including puddle , Hendrickson / Klughammer and Kramer protocols. Protocol measurement parameters are provided along with the quenching relaxation parameters q_E, q_M, q_Z, q_T, and q_I. While originally designed for the puddle model, the resurrection of NPQ in Klughammer lake model parameters allows work in the area of quenching relaxation using the Hendrickson / Klughammer lake model parameters as well. There has been significant work in this direction. By taking the work done by Hendrickson (2004), a group of well known researchers has further divided lake

model NPQ into q_E, q_T, and q_I. (Ahn T.K, Avenson T.J. Peers G. Dall'Osto L., Bassi R. Niyogi K.K. Fleming G R. 2008). These relaxation quenching parameters have value in the study of state transitions and photoinhibition as separate phenomenon. q_M is chloroplast migration (Cazzaniga 2013), and q_Z is a proposed unknown xanthophyll mechanism (Nilkens 2010).

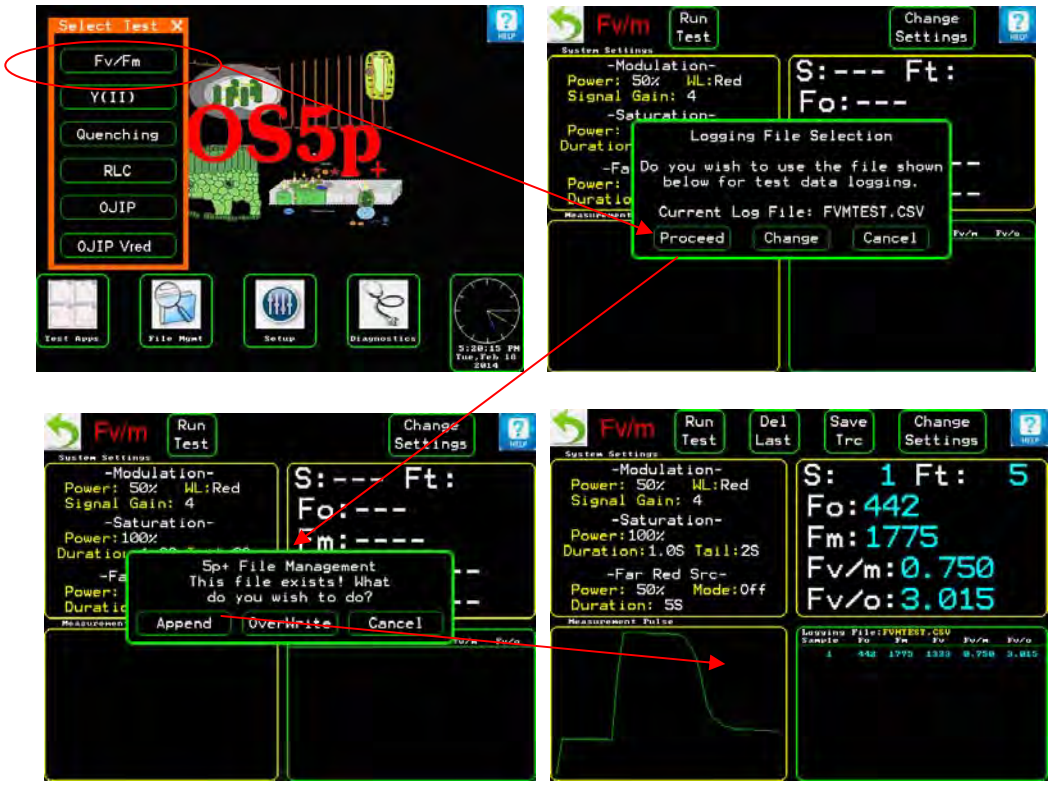
q_E, q_M, q_Z, q_T, and q_I are also displayed when using Kramer equations in the relaxation test. They are derived from NPQ, a parameter that is a Hendrickson / Klughammer parameter and not a Kramer parameter, The relaxation parameters are available for comparison purposes.

4. Rapid light Curves (RCL) – allows the stepping of actinic light up and down during a test. This test is commonly used to study saturation characteristics of plants especially for under canopy work or for aquatic plants. While almost all chlorophyll fluorescence measuring parameters required steady state photosynthesis for reliable measurement, this protocol is an attempt to allow measurement of PSII under changing light conditions.
5. OJIP. This is the Strasser protocol OJIP used for plant stress testing. The most used Strasser parameters are displayed on the measuring screen and all of the Strasser parameters are reported to the data file.

OJIP Vred. Is a special OJIP quenching protocol designed in conjunction with Wim Vredenberg. It is designed to allow investigation of quenching phenomenon with high speed time resolution, and measurement of saturation pulse NPQ after microseconds, milliseconds or seconds.

Running the F_V/F_M Test

The last file used for this type of measurement will open up and a small screen will appear within the larger screen. It will show the file name and ask if the file is to be used and if one wants to **"Proceed"** measuring with this file and the parameters that have been set. The other options are to **"Change"** and create a new file, or touch **"Cancel"** to go back to the main menu.



Tapping **"Append"** allows additions to an existing file, **"OverWrite"** erases the last measurement and allows new additional measuring data to replace it with the same instrument settings, and **"Cancel"** sends you back to the main menu.

F_V/F_M measuring Screen

Change Settings screen

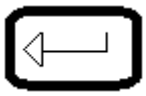
Loading and Saving Preset Measuring Routines

"Load Presets" and **"Save Presets"** The OS5p+ provides two types of files, preset files and data files. Measuring parameters, once set, are stored as preset files by tapping "Save Presets"**.** To recall saved Preset files, tap "Load Presets". Data files are created, recalled, and appended when the quenching protocol is opened or under **Data Management "Log file Name"** If Measuring parameters are changed while using a specific data file, the new parameters are listed above the data in the data file, collected after the change, automatically. When saving a set of measuring parameters to a preset file, tap the "Save Preset" button and the options "New", "Existing" or "Cancel" will appear.

If **"New"** is selected, an alpha-numeric keyboard will appear and allow an 8 character name to be input. When finished, tap the return key to save the file. The suffix for Preset files is .FVM (for FV/FM, .YLD (for Y(II)), .KIN (for quenching), .OJP (for Strasser OJIP), .FIP (for Vredenberg OJIP protocol). The suffix for data file names is .CSV .

If **"Existing"** is selected, a previously made list of Preset files will appear. Select a file to be modified, or tap the "X" on the top right hand side of the orange box to cancel. If one has important Presets files or data files, copies should be saved to a data card for future retrieval. This way, if multiple researchers are using an instrument, the important measuring parameters and data can be saved safely.

When the parameters have been adjusted or changed for a specific set of conditions, the test setup can be saved by pressing "Save Preset" while in the F_V/F_M setup screen. A key board screen will appear, and the test set up can be named and it is saved when the return key is tapped on the key board. To exit the screen tap the return arrow in the upper left of the screen.



Return key

To load previously created tests, press "Load Presets". Find the file of interest and tap the file of interest to load it. Press the test of interest. After it is highlighted, tap the name of the file. The number of tests that can be saved is almost unlimited. Touch the top of the list to scroll

up and the bottom of the list to scroll down. Exit if there is no action by tapping the "X" in the upper right hand corner of the orange box.

To set up the F_V/F_M test, tap the **"Change Settings"** button. The Set up screen will appear.

The section under **Modulated Light** is for adjustment of the measuring light. This light turns on and off rapidly. The frequency for each test is set at the factory to ensure reliable operation automatically.

The OS5p+ provides a red modulated light source and a blue modulated light source. Select the type of light source that you want to use under **"Wavelength"**. Red is the most used light source. Blue may be used for cyano-bacteria some algae and for other application. If you do not know which light source to use, it is likely that red is the best choice.

For F_V/F_M, the **"Intensity"** must be high enough to measure a fluorescence signal but not too high. If it is too high it will become actinic and cause a partial reduction of Q_A. If this happens, an error will result that reduces F_V/F_M. The correct setting will allow a fluorescence signal measurement without the reduction of any Q_A.

The OS5p+ has an automated routine that ensures that the modulated light intensity and the gain are set just right. Place a dark clip on the type of leaf that will be used. Insert the fiber optic into the opening of the dark clip, and then open the dark slide. The leaf will be exposed to the existing modulated light level. Next tap the **"Auto Setup button"** in the set up screen. Beeps will sound while it automatically adjusts the light source so that the intensity and gain are optimal. This is done to help ensure error free operation.

The **Saturation Light** section is used to adjust the saturation light. The intensity can be set by tapping the **"intensity"** button. 100% represents 15,000 μmols of irradiation.

The **"Flash Width"** or saturation pulse duration may be set between 0.4 seconds and 1.5 seconds. Opti-Sciences has also semi-automated this function to reduce measuring errors. An algorithm is provided, in all of the modulated protocols, that uses an 8 point 25 ms. rolling average to detect the highest measuring points for F_M, and F_M', this eliminates saturation pulse NPQ from being a problem. This means, that as long as the saturation pulse is wide enough, the optimal answer will be provided regardless of saturation pulse NPQ. The literature states that land plants have an optimal range of 0.5 seconds to 1.5 seconds. For Algae the optimal times are shorter, they range from 25 ms. to 50 ms. Saturation pulse NPQ is a rounding of the tail end of the saturation pulse fluorescence signal that happens if the flash width is too wide. In previous systems that would average the entire top of the flash this rounding would cause a measuring error. (Roseqvist and van Kooten 2006).

The **"Flash Tail"** allows viewing of the saturation flash tail at the end of the test, on the graphic screen. If there is no tail then just the rise is shown. It does add to the measuring time.

Far red light can be used to ensure complete re-oxidation of PSII by stimulation of PSI. By pre-illumination of the leaf with far red light for 5 to 10 seconds (Maxwell & Johnson 2000). Under far red light, PSI takes the remaining electrons from PSII, ensuring a more fully oxidized PSII. Test have been done that show that 10 seconds is better than 5 seconds; however, this does add another 5 to 10 seconds to the measuring time. Far red light does not allow the elimination of dark adaptation (Consalvey (2004). The pH of the thylakoid lumen, the xanthophyll cycle, chloroplast migration, and state transitions (where they exist), must all relax for proper measurement. The **"Intensity"** can be set from 0 to 100%. The **"Duration"** can be set from 1 to 15 seconds. The **"Mode"** button is used for pre-illumination, off, or on during the entire measurement.

Data Management

"Log File Name" If this button is tapped a screen will appear that offers **"New"**, **"Existing"**, or **"Cancel"** options. If New is selected, then a screen with **"New"**, **"Kwik Name"**, or **"Cancel"** can be selected. If **"New"** is tapped on this screen, an alpha numeric keyboard will appear and an 8 character name may be input. Tap the return key to save the name. If **"Kwik Name"** is tapped, today's date and time become the new file name. If "Cancel" is tapped, the instrument goes back to the previous screen. If **"Existing"** is tapped, a list of existing files with previously set parameters appears. By tapping one of these file names, the file and the parameters that were created earlier will be loaded into the active memory and used for measurement.

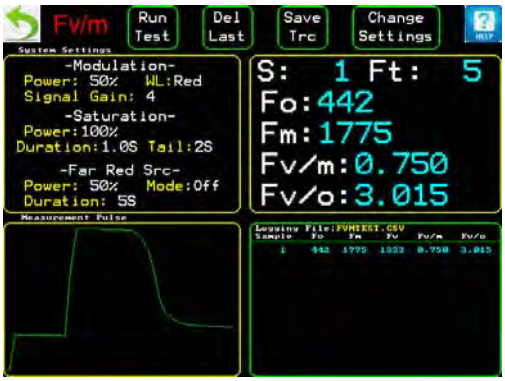
"Add a Note" To add a note for a specific measurement, tap this screen. An alpha numeric keyboard will appear and a 34 character note may be entered into the data file. Tap the return key to save the note.

The number of the measurement may be changed under **"Change Sample #"**

When done, tap **"Save Presets"** to save the settings. A small screen will appear asking if the changes should be made to a new file, the existing file, or cancel the operation.

To load Presets from an existing file, tap **"Load Presets"**. A list of files will appear and one can be selected.

Tap the Arrow in the upper left hand corner to go back to the measuring screen.



From the measuring screen, Options include **"Save a Measuring Trace"**, **"Delete the Last Measurement"**, or **"Run the Test"**.

The window on the lower right hand side will show results of measurements that have been made. One can **scroll** through a long list by taping on the bottom of the list, or near the top of the list.

Data logging is automatic. Unless a measurement is deleted, it is automatically saved to the data file. The trace must be saved individually by tapping the **"Save Trc"** button.

Help Screen

The Question mark button is a help screen that provides basic instructions for the screen shown. It is for future development. Use this manual for questions, or contact Opti-Sciences Inc.

Error messages common to the test modes:

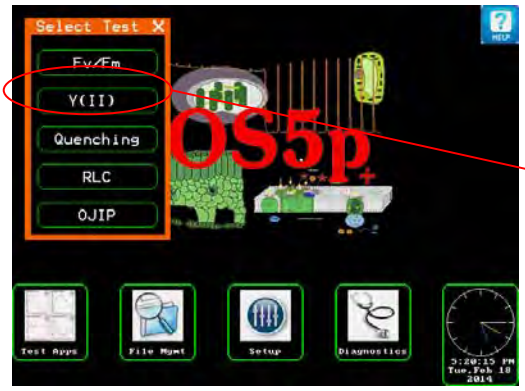
There are several common errors that can occur in the test modes.

If the battery voltage is 10.5 or less, the warning message "!! Battery Low !!" will appear and a long beep will be sounded.

If the amount of in-band IR radiation is above set limits thereby saturating the pre-detector the error message "!! IR to High !!" will be displayed.

"Fluorescent signal too low" appears if there is not enough signal getting to the detector. This may be due to the fiber optic being too far from the sample, the intensity of the modulated source being too low or the gain control set too low.

Y(II) Protocol: Quantum Yield of PSII - Y(II) or ΔF/F'



Quantum Yield of PSII -Y(II) (or $\Delta F/F_M'$, or $F_M'-F_S/F_M'$) (Genty 1989) is a fast (it normally takes about two seconds) light adapted, steady-state light adapted test, that provides a measure of the proportion of the amount of light used in photochemistry in PSII to the amount of light absorbed by chlorophyll associated with PSII. (Maxwell and Johnson 2000). It can also be viewed as achieved efficiency of photosystem II under current steady-state photosynthetic lighting conditions with quenching mechanisms at steady-state photosynthesis. It is the easiest test to take because the test sample does not have to be dark adapted. Samples are tested under steady state photosynthetic conditions with ambient light exposing the sample while the measurement is taking place. Modulated fluorometer design allows this measurement.

This is different from F_V/F_M, a dark-adapted measurement, that measures maximum quantum efficiency. F_V/F_M does not measure achieved photosynthetic efficiency while photosynthesis is taking place.

Quantum Yield of PSII provides a normalize ratio that is a related to electron transport and Carbon assimilation, or achieved photosynthesis efficiency. Under laboratory conditions, and most stress conditions, Yield of PSII provides a measure directly related to linear electron transport and correlates very well with carbon assimilation. It has been found to correlate to carbon assimilation in a linear manner for C_4 plants, and in a curve linear manner for C_3 plants. (Genty 1989, 1990). Early correlation to carbon assimilation can be reduced for C_3 plants due to photorespiration, pseudo-cyclic electron transport, and possibly other electron sinks. This does not occur in C_4 plants because they have no significant photorespiration. For more information regarding the types of plant stress that are photo respiratory, review the Opti-Sciences Plant Stress Guide.

In the Y(II) test App or protocol, it is assumed that the sample is being illuminated by an actinic light source to drive photosynthesis. Either the sun or an artificial light can be used. The OS5p+ has a white light diode with an intense blue spectrum that is used to provide a more natural actinic light source and allow chloroplast migrations to occur as they do in nature (Cazzaniga 2013). It can be used to pre-illuminate leaves with the PAR Clip at a stable adjustable actinic level. The PAR clip maintains actinic light levels up to 1,800 µmols.

Yield of PSII, Y(II), has been shown to measure plant stress more successfully and earlier than F_V/F_M with some types of plant stress. For example Y(II) can be used more effective

when measuring heat stress and water stress. (See the Desk top plant stress guide for more details (www.optisci.com .)

It is imperative that the light level is measured and controlled. When the PAR Clip is clamped on the leaf, the angle of the leaf should not be changed and the measuring area should not be shaded. The leaf is at steady state before clamping on a sunny day, and changing leaf orientation or shading can cause an error. Only samples with very similar PAR values should be compared, and samples with similar light histories. Sun leaves should be compared with sun leaves. The photosynthetic composition of sun leaves and shade leaves are different. With this in mind, a PAR Clip is highly recommended for all Y(II) measurements.

Steady State photosynthesis is an equilibrium of various plant mechanisms at a specific light level. Factors that determine the time required for steady state photosynthesis to be reached include the xanthophyll cycle, Δph of the thylakoid lumen, chloroplast migration, and state transitions where they exist. The xanthophyll cycle, Δph of the thylakoid lumen typically reach steady state in a few minutes, and state transitions can take fifteen to twenty minutes. Maxwell and Johnson (2000) found that it took fifteen to twenty minutes for 22 British terrestrial plants to reach steady state photosynthesis. However, new research shows that it takes between twenty minutes to thirty five minutes for migration of chloroplasts in dicots. Since the fluorescence measurement of q_M or chloroplast migration represents about 30% of NPQ at high light levels, it must be considered (Cazzaniga 2013). This research show the fluorescence change thought to be caused by state transitions, at least at higher light levels, is due to chloroplast migration.

Furthermore high white light or high blue light intensity is required to trigger chloroplast migration. High red light does not cause significant chloroplast migration as found in nature (Cazzaniga 2013). The OS5p+ has a white light LED light source that contains an intense blue spectrum, ideal for chloroplast migration and pre-illumination at a set light level.

As with the "F_V/F_M" test, far-red pre-illumination is an option. Far –red illumination may also be turned off or turned on during the entire measurement. If ambient sun light is used for yield of PSII measurement, far-red light is provided by sunlight. It can be added in the set up menu if internal artificial illumination is used to drive PSI . Internal actinic illuminators normally filter out far red light for greater measurement control.

When the test is triggered, F_s (or F'), the measure of steady state fluorescence is determined and then the saturation pulse is applied to close all capable PSII reaction centers for determination of F_M'. In steady state photosynthesis, F_M' is depressed from F_M by mechanisms grouped in non-photochemical quenching. This group included mechanisms that are really something else, but they are included in the group anyway. Things like chloroplast migration, state transitions, and photoinhibition are included in this list.

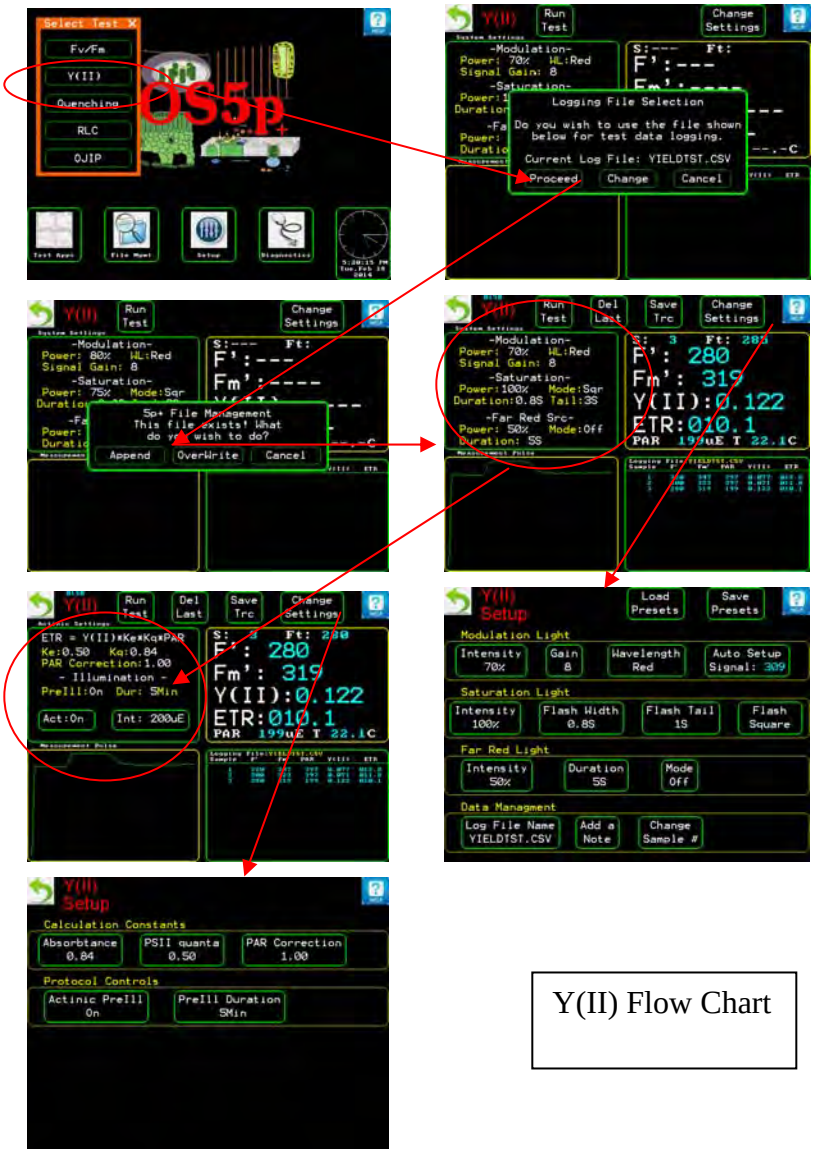
The resultant data is then processed to find Y(II), (or $F_M' - F'/F_M'$). A graph of the measured data is displayed along with measured and calculated parameters. ETR or relative electron transport rate and leaf temperature are also displayed if the measurement is made with a PAR clip. PAR Clips are standard with the OS5p+.

"Default" PAR values can be put in the measuring file as well as measured values. This is done on the test Setup screen. "Default" PAR is only used when the PAR clip is not used. However, the importance of PAR measurement can not be overstated.

When the PAR Clip is not used, this number sets the PAR reading. It has a range from 0 to 2496 µmol in steps of 1 µmol (1 µml), and it allows estimated ETR. If the PAR Clip is used, then the measured PAR value is always automatically reported to the measuring file.

Running the Y(II) Test

From the main screen select **"Test Apps"** by pressing the touch screen. The Test Selector menu will appear. Press the "Y(II)" button.



Y(II) Flow Chart

Next, one can choose to proceed with the last measuring file and set of instrument settings that were used by tapping **"Proceed"**. If **"Change"** is tapped it, is possible to select another measuring file, create a new one, or the process may be canceled by tapping **"Cancel"**. If cancel it pressed, the instrument goes back to the previous screen.

In the Y(II) measuring screen, a green square means that more information can be seen by touching that green window. The **"Change Settings"** button allows the settings displayed in the upper left hand corner window to be changed. For other parameters, tap the window in the upper left hand corner first.

Y(II) Measuring Screens

When "Proceed" is pressed, there are three choices. **"Append"**: Add measurements to the existing file. **"Cancel"**: Go back to the main menu; and **"OverWrite"**: Use the file that is currently being used but the new measurement will overwrite the previous measurement.

To measure, tap **"Run Test"** on the measuring screen. To delete the last measurement, tap **"Del Last"**. To save the fluorescence measuring trace seen in the lower left hand corner of the measuring screen tap **"Save Trc"**. The trace will not be saved in the data file unless this is done.

Y(II) set up screen reached from the measuring screen window by tapping "Change Settings" when the upper left hand window lists -Modulation-

Y(II) set up screen reached from the measuring screen window by tapping "Change Settings" when the upper left hand window lists:

ETR = Y(II) * Ke * Kq * PAR

Loading and Saving Preset Measuring Routines

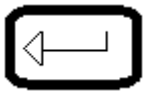
"Load Presets" and **"Save Presets"** The OS5p+ provides two types of files, preset files and data files. Measuring parameters, once set, are stored as preset files by tapping "Save Presets"**.** To recall saved Preset files, tap "Load Presets". Data files are created, recalled, and appended when the quenching protocol is opened or under **Data Management "Log file Name"** If Measuring parameters are changed while using a specific data file, the new parameters are listed above the data in the data file, collected after the change, automatically. When saving a set of measuring parameters to a preset file, tap the "Save Preset" button and the options "New", "Existing" or "Cancel" will appear.

If **"New"** is selected, an alpha-numeric keyboard will appear and allow an 8 character name to be input. When finished, tap the return key to save the file. The suffix for Preset files is

.FVM (for FV/FM, .YLD (for Y(II)), .KIN (for quenching), .OJP (for Strasser OJIP), .FIP (for Vredenberg OJIP protocol). The suffix for data file names is .CSV .

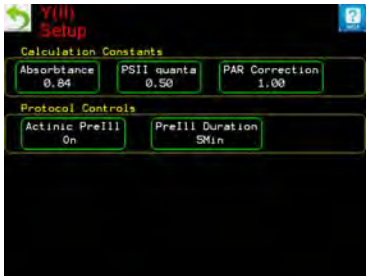
If **"Existing"** is selected, a previously made list of Preset files will appear. Select a file to be modified, or tap the "X" on the top right hand side of the orange box to cancel. If one has important Presets files or data files, copies should be saved to a data card for future retrieval. This way, if multiple researchers are using an instrument, the important measuring parameters and data can be saved safely.

When the parameters have been adjusted or changed for a specific set of conditions, the test setup can be saved by pressing "Save Preset" while in the Y(II) setup screen. A key board screen will appear, and the test set up can be named and it is saved when the return key is tapped on the key board. To exit the screen tap the return arrow in the upper left of the screen.



Return key

To load previously created tests, press "Load Presets". Find the file of interest and tap the file of interest to load it. Press the test of interest. After it is highlighted, tap the name of the file. The number of tests that can be saved is almost unlimited. Touch the top of the list to scroll up and the bottom of the list to scroll down. Exit if there is no action by tapping the "X" in the upper right hand corner of the orange box.



Y(II) set up screens (see the previous pages for moving from one to another)

Modulation Light

"Intensity" It may be set manually from 0 to 100%. This is the measuring light. It allows measurement of FO before any Q_A has been reduced and it allows Y(II) to be measured in a light adapted environment.

"Gain" is the electronic gain of the photodiode sensor. It may be manually set. Lower settings have less electronic noise, and higher settings allow lower level fluorescence detection.

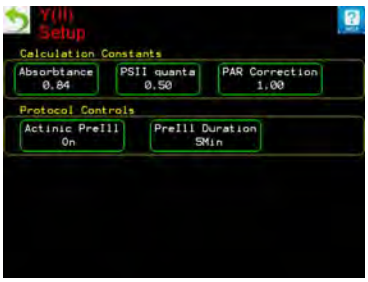
"Wavelength" The OS5p+ provides both a red and a blue modulated light. Touch the button to switch sources. Red is the most commonly used light source with blue being used for specialized applications such as for cyanobacteria, and some algae work.

"Auto Setup" This button allows auto setup of the modulated light intensity and gain. It sets the modulated light and gain high enough to measure a fluorescence signal but not high enough to drive photochemical reduction of any Q_A. It is designed to reduce mistakes.

Saturation Light

"Intensity" It may be set from 0 to 100%. At 100%, using the PAR clip, the intensity at the leaf will be 7,500 µmoles with a square flash, and 7,000 µmoles with the Loriaux 2013 multiple-phased saturation flash, as optimally indicated by the research (Called Multiflash).

"Flash Width" This may be set from 0.4 to 2.0 seconds. The optimal width varies by species. Land plant optimal settings vary from 0.5 seconds to 1.5 seconds. Algae vary from 25 ms. to 50 ms. Opti-Sciences includes a special algorithm that eliminates this as a source of error as long as the saturation flash is wide enough. It uses a 25 ms 8 point rolling detection average to determine the highest F_M, or F_M' regardless of saturation pulse NPQ. Therefore, it can be used for algae work without concern. For this reason, the default value is set at 0.8.



Y(II) set up screens (see the previous pages for moving from one to another)

"Flash Tail" This can be set from 0 to 5 seconds. It allows graphic imaging of the tail end of the saturation flash and the resulting saturation flash NPQ for up to 5 seconds. If it is set to 0 then just the rise and top of the saturation flash will be graphically displayed. Extending the flash tail display increases measuring time.

"Flash" The choices here are the standard square top flash listed as **Square**, and **MultiLvl.** The multiLvl is something we call Mutiflash, or a multiple-phased single saturation flash according to the research of Loriaux 2006 and Loriaux 2013. The protocol follows the latest recommended Loriaux 2013 protocol. The Initial flash is 7,000 mols for 0.3 seconds, the down ramp is 20%, the down ramping rate is less than 0.01 mol photons $m^{-2}s^{-2}$, and then the final intensity flash is at 7,000 mols again for 0.3 seconds, to check for saturation flash NPQ (Loriaux 2013). It has been found that under near saturating light conditions that even the most intense saturation flash will not close all or the available reaction centers, causing an error. The Loriaux 2013 method corrects for this error by using least squares linear regression to determine Y(II) and ETR with an infinitely intense saturation flash. This is not an issue at lower light levels. The Multiflash may be used in the Y(II) protocol or in all quenching protocols if it is selected.

Far red light

"Intensity" Far red light is used in the Y(II) protocol when the sample is pre-illuminated with the internal white light actinic light source. Far red light stimulates PSI, and it is found in sun light so there is no reason to use it for field work. Far red light is intentionally filtered out of the white actinic light source to allow more control over measurement. One should consider using the far red light when pre-illuminating samples using the built in stable white actinic light source. Set the intensity to 100%.

"Duration" The duration may be set from 0 to 5 hours.

"Mode" This may be set to off, on or pre. Using pre, will pre-illuminate the sample before the saturation pulse or flash.

Y(II) set up screens (see the previous pages for moving from one to another)

Data Management

"Log File Name" If this button is tapped a screen will appear that offers **"New"**, **"Existing"**, or **"Cancel"** options. If New is selected, then a screen with **"New"**, **"Kwik Name",** or **"Cancel"** can be selected. If **"New"** is tapped on this screen, an alpha numeric keyboard will appear and an 8 character name may be input. Tap the return key to save the name. If **"Kwik**

Name" is tapped, today's date and time become the new file name. If **"Cancel"** is tapped, the instrument goes back to the previous screen. If **"Existing"** is tapped, a list of existing files with previously set parameters appears. By tapping one of these file names, the file and the parameters that were created earlier will be loaded into the active memory and used for measurement.

"Add a Note" To add a note for a specific measurement, tap this screen. An alpha numeric keyboard will appear and a 34 character note may be entered into the data file. Tap the return key to save the note.

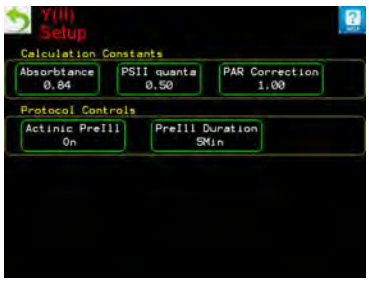
"Change Sample #" This allows the sample # in the measuring file to be changed.

Calculated Constants

"Absorptance" 0.84 is an average leaf absorptance measurement used to provide an average value. Actual leaf absorptance varies from 0.7 to 0.9 in healthy plants using white light. It also changes for parts of the visible spectrum, plant stress, growing conditions, chlorophyll content, leaf age, species, and light level. It is used in the equation for ETR or electron transport rate.

" PSII quanta" This is the ratio of PSII reaction centers to PSI reaction centers. Thee average here is 0.5; however, in C_3 and C_4 land plants, the range is from 0.4 in some C_4 plants to as high as 0.6 in some C_3 plants. It varies with plant type (C_3, C_4), species, lighting during growing conditions, and under severe carbon deficits. It is used in the equation for ETR or electron transport rate.

"PAR Correction" This is a catch all correction. Normally it is set to one, however, Rasher (2000) found that there is a PAR sensor location error when using an internal illuminator with a PAR sensor. He found that PAR value was about 10% less at the leaf using his PAR clip and system. The PAR correction factor allows correction of that distance if necessary. The correction range is between 0.01 to 2.00. The default setting is 1.0.



Y(II) set up screens (see the previous pages for moving from one to another)

Protocol Controls

"Actinic PreIll" This function allows the use of the internal stabilized white light LED to be used for pre-illumination of the sample. Instead of living with the solar intensity at any given time, this allows pre-illumination at a specific selected intensity. It is held stable using a feed back loop in conjunction with the PAR sensor on the PAR clip. The PAR clip may be used in a darkened room or covered with a black shroud. The actinic light source is a white light LED with an intense blue spectrum to ensure proper chloroplast migration as found in nature. At high light levels, chloroplast migration is responsible for about 30% of NPQ in tested samples. Intensities may be set from 0 to 2000 µmols.

"PreIll Duration" This may be set from 0 to 5 hours. For the longest times, a battery belt or AC current may be needed under extreme heat or cold conditions.

Multi-flash "MultiLll"

Standard single square topped saturation pulse or Multiflash

Saturation flashes used with modulated fluorometers are designed to close all PSII reaction centers.

The resulting maximum fluorescence intensity value, of the saturation flash, is used in most measurements including, quantum yield of PSII (also called Y(II) or ΔF /FM', or ΦPSII), ETR (or J), and quenching parameters all use F_M' in their calculations.

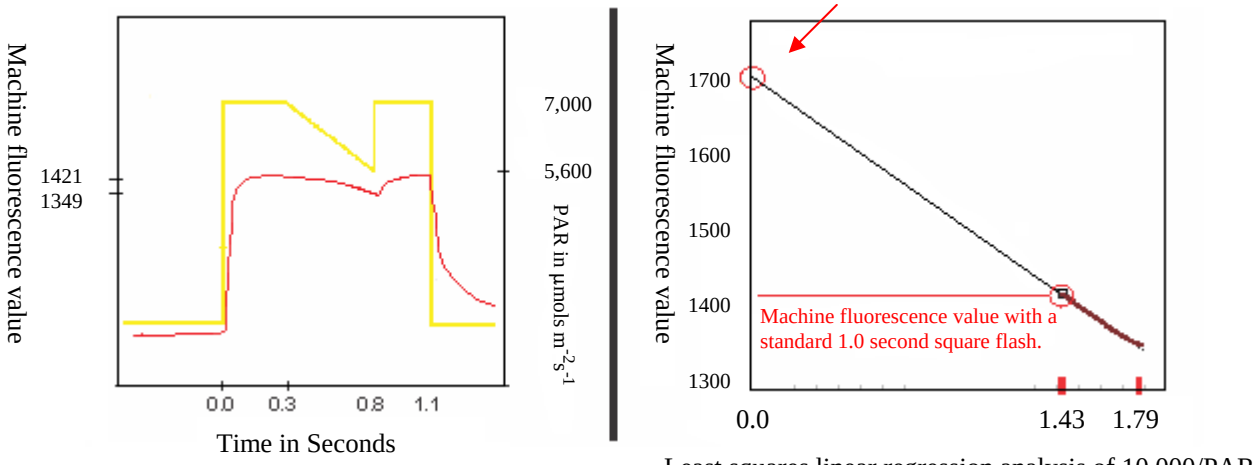
While it is possible to close all reaction centers in a properly dark adapted sample, with a relatively low amount of light, it has been found that in light adapted samples, with a high actinic light history, complete closure of all PSII reaction centers becomes problematic with even highest amounts of saturation light. In a 2006 poster and in a 2013 paper (Loriaux 2013) researchers that included Bernard Genty, the developer of quantum yield of PSII (or) ΔF /FM') protocol, verified the issue, and developed a method for F_M' correction. The method involved a single multiple phased saturation flash. It starts at 7,000 µmols for about 0.30 seconds, and then the saturation flash intensity is ramped down 20% over another 0.5 seconds, in a linear fashion, while recording a high number of chlorophyll fluorescence values along the way. The ramping rate is less than 0.01 µmols $m^{-2}s^{-1}$. The final phase lasts about 0.3 seconds, finishing at the original 7,000 µmols, to detect any saturation flash NPQ.

Least squares linear regression analysis of 10,000 / PAR, is then used to determine the F_M' fluorescence level with an infinitely intense saturation flash. Research has shown that Y(II) measurements, taken under high actinic light conditions, can be underestimated with up to a 22% error, and there can be up to a 41% error in ETR values if this method is not used.

From a historical point of view, studies by Earl (2004), Loriaux (2006), and Loriaux (2013) have compared chlorophyll fluorescence measurement results with gas exchange measurements and found that by using multiple saturation flashes, and regression analysis, an infinite fluorescent saturation light flash intensity can be determined and used to correct Y(II) and ETR measurements. Loriaux 2006 and 2013 developed a single multiple phased saturation flash to do the same thing. Opti-Sciences provides this method for F_M' correction, called Multi-flash. This standard option is available in all measuring protocols, including quenching measurements and rapid light curves, and it can be turned off or on. The standard square flash may also be used. The Loriaux 2013 method is the most a highly accepted method, with the highest degree of accuracy. Accuracy is improved by about 3% over the Loriaux 2006 method.

When using a standard square saturation flash, the flash duration and intensity may be adjusted. In addition, we use an eight point rolling average over a 25 ms time frame to determine maximum F_M and F_M' values with the square flash to eliminate any saturation flash NPQ errors. With Multiflash, The saturation duration is always 1.1 seconds, the maximum intensity is always 7,000 µmols $m^{-2}s^{-1}$, and the down ramp is always 20% at a rate less than 0.01µmols $m^{-2}s^{-1}$. This is the configuration that Loriaux 2013 paper found provided optimal results.

Representation of how Multi-Flash Works / Regression Analysis Graph



Least squares linear regression analysis of 10,000/PAR

The “Multi-flash” option may be turned on or off in the Yield Protocol by pressing the window in the upper left had corner that displays the –Modulation- title.

Under “Flash” select “MultiLll”. For using the Multiflash protocol and select “Square” for standard single square topped saturation pulse method.

Measurement Review

On the main Y(II) measuring screen, the lower right hand side logs all of the measurements made in the current measuring session for the current file name. One can scroll through measurements by touching the screen. If one touches the top of the review area, the earliest measurements may be reviewed. If one touches the bottom of the review area, then the latest measurement can be reviewed. The session remains current as long as the file name remains the same and one does not leave the Y(II) protocol. It will stay intact if one goes to different screens within the Y(II) protocol. If one turns off the instrument, changes the file name, or goes to a different protocol, then the session is ended. All measuring data is retained with the current file name in the measuring file, and it may be viewed in Excel on a computer. It does not disappear like the measurement review session data.

Help Screen

The Question mark button is a help screen that provides basic instructions for the screen shown. It is for future development. Use this manual for questions, or contact Opti-Sciences Inc.

Error messages common to the test modes:

There are several common errors that can occur in the test modes.

If the battery voltage is 10.5 or less, the warning message “!! Battery Low !!” will appear and a long beep will be sounded.

If the amount of in-band IR radiation is above set limits thereby saturating the pre-detector the error message “!! IR to High !!” will be displayed.

“Fluorescent signal too low” appears if there is not enough signal getting to the detector. This may be due to the fiber optic being too far from the sample, the intensity of the modulated source being too low or the gain control set too low.

Relative Electron Transport Rate

Relative Electron Transport Rate –
ETR = Y(II) * PAR * .84 (leaf absorptance) *.5 (ratio of PSII to PSI reaction centers)

Average plant values are used in the standard equation. 0.84 is a good average value for many species of plant and conditions (Bjorkman and Demming, 1987), research has shown that the leaf absorption coefficient can vary between 0.7 and 0.9 in healthy plants using a red modulated light source (Eichelman H. 2004). Absorptance varies with species, chlorophyll content, leaf age, plant stress, and by light level (light level - see Cazzaniga 2013). Note: When using the blue modulated light it was found that the average absorption value should be set closer to 0.94 instead of 0.84. Leaf absorptance is usually measured using an integrated sphere, a light source and a spectrophotometer.

Research has also shown that the fraction of light that is absorbed by PSII reaction centers varies by species, plant type (C_3, C_4 plant or other), sun or shade growing conditions, and under severe carbon deficit. species and can range from at least .40 to .60 (Laisk and Loreto,1996). Some C_4 plants like corn are close to 0.40 and some C_3 plants can be closer to 0.60. While 0.5 is used for an average value, the most used method for measuring the ratio of PSII to PSI, involves the use of spectral analysis of samples at 77^{o}K (Anderson 1999), (Zell 2010);

Baker (2008) states the ETR should not be used for comparing different samples unless leaf absorptance, and the ratio of PSII to PSI reaction centers have been measured. For this reason, Y(II) is more properly used for plant stress measurement.

The ratio of PSII reaction centers can be changed by pressing **“PSII quanta”** and using the key board screen. Similarly, the over all leaf absorption can be changed by pressing **“Absorptance”**. It has been made available for more exacting work when necessary.

PAR Correction is for correcting PAR sensor location error according to Rascher (2000). When some artificial light sources are used, Rascher found that the location the PAR sensor relative to the leaf surface can cause an error of up to 10%. This error is insignificant if sun light is used due to the much greater distance from the light source. Rascher used an independent PAR sensor and measured the intensity at the leaf plane. He then made Corrections due to PAR Clip sensor location by comparing the differences between the PAR clip values and the leaf plane values. This correction is not needed for most relative comparison ETR applications, however it has been made available for more exacting work when necessary.

By plotting ETR vs. PAR, potential ETR rates can be determined, photosynthetic capacity, as well as ETR rate limitations at given light intensities or leaf temperatures. (U. Schreiber 2004). Note: Four electrons must be transported for every CO_2 molecule assimilated or O_2 molecule evolved.

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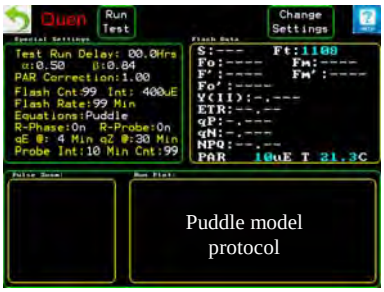
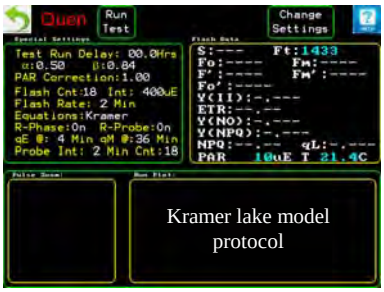
Running quenching measurements

Kramer lake model, Hendrickson – Klughammer lake model,

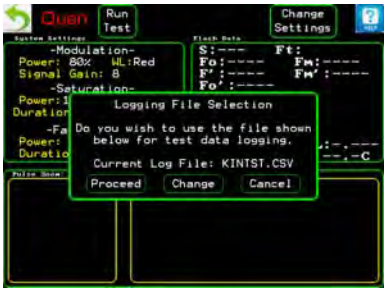
& puddle model quenching

Quenching Flow chart





Flow chart for quenching measurements continued



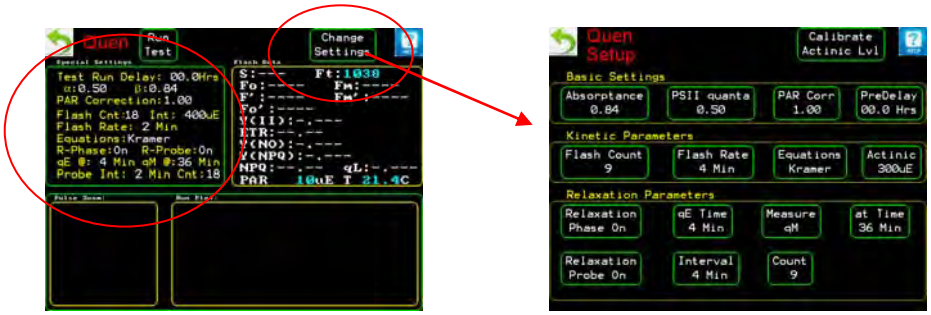
Tap the **"Test Apps"** icon above with your finger and an orange box appears.

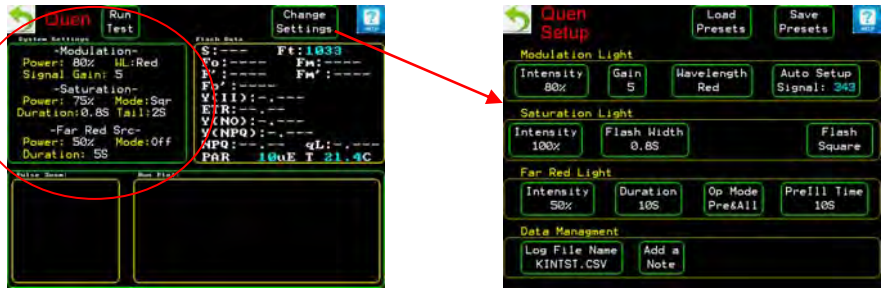
Tap the Quenching button with your finger.

The logging file selection box appears. If **"Proceed"** is selected then the 5p+ File Management screen appears. If **"Change"** is selected, then a key board appears to change the file name. If **"Cancel"** is selected then it goes back to the main OS5p+ screen.

In the 5p+ File Management screen, **"Append"** adds to the last used existing measuring file, and the main quenching measuring screen appears. If **"OverWrite"** is tapped it will over write the last measurement made. If **"Cancel"** is tapped the instrument goes back to the previous screen.

On the main quenching measuring screen shown below on the top left, there are several choices that can be made.





"Load Presets" and **"Save Presets"** The OS5p+ provides two types of files, preset files and data files. Measuring parameters, once set, are stored as preset files by tapping "Save Presets". To recall saved Preset files, tap "Load Presets". Data files are created, recalled, and appended when the quenching protocol is opened or under **Data Management "Log file Name"** If Measuring parameters are changed while using a specific data file, the new parameters are listed above the data in the data file, collected after the change, automatically. When saving a set of measuring parameters to a preset file, tap the "Save Preset" button and the options **"New"**, **"Existing"** or **"Cancel"** will appear.

If "New" is selected, an alpha-numeric keyboard will appear and allow an 8 character name to be input. When finished, tap the return key to save the file. The suffix for Preset files varies with the measuring protocol, the suffix for data file names is .CSV .

If "Existing" is selected, a previously made list of Preset files will appear. Select a file to be modified, or tap the "X" on the top right hand side of the orange box to cancel. If one has important Presets files or data files, copies should be saved to a data card for future retrieval. This way, if multiple researchers are using an instrument, the important measuring parameters and data can be saved safely.

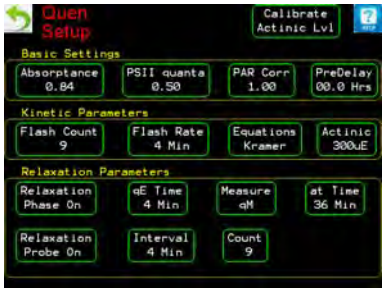
Parameters in the upper left hand corner window can be changed by tapping on the **"Change Settings"** button. To change other parameters, tap the window in the upper left hand corner and more parameters will appear. When these parameters appear in the window, they may be changed by tapping on the "Change Settings" **b**utton.

Quenching setup Page 1

Basic Settings

Basic Settings include the leaf **"Absorptance"** used in the ETR or (electron transport rate) equation, **"PSII quanta"** or the ratio of PSII to PSI reaction centers used in the ETR equation, **"PAR Corr"** a value that allows one to adjust for PAR sensor location error (As per Rascher 2000), and **"PreDelay"** a dark adaptation timer that allows dark adaptation for a specific period of time up to 25 hours.

"Absorptance" 0.84 is an average leaf absorptance measurement used to provide an average value. Actual leaf absorptance varies from 0.7 to 0.9 in healthy plants using white light. It also changes for parts of the visible spectrum, plant stress, growing conditions, chlorophyll content, leaf age, species, and light level (for light level change use Cazzaniga 2013) (Baker 2008 for the other sources of change). It is used in the equation for ETR or electron transp



Calibrate Actinic Lvl may or may not be used. The PAR clip will adjust to any programmed light level when the PAR Clip is used. However, if the Calibrate Actinic light button is tapped before it attached to the sample, it finds the program actinic value before the quenching test and jumps to the programmed actinic light intensity more quickly than if it is not used.

" PSII quanta" This is the ratio of PSII reaction centers to PSI reaction centers. Thee average here is 0.5; however, in C_3 and C_4 land plants, the range is from 0.4 in some C_4 plants to as high as 0.6 in some C_3 plants. It varies with plant type (C_3, C_4), species, lighting during growing conditions, and under severe carbon deficits. It is used in the equation for ETR or electron transport rate. (Baker 2008 is the source of PSII ratio change)

"PAR Correction" This is a catch all correction. Normally it is set to one, however, Rasher (2000) found that there is a PAR sensor location error when using an internal illuminator with a PAR sensor. He found that PAR value was about 10% less at the leaf using his PAR clip and system. The PAR correction factor allows correction of that distance if necessary.

The correction range is between 0.01 to 2.00. The default setting is 1.0. (Rasher 2000 is the source for a need for PAR correction)

"PreDelay" a dark adaptation timer that allows dark adaptation for a specific period of time up to 25 hours. It is designed to offer greater control and greater automation in quenching measurements. It is common to dark adapt overnight for quenching field measurements. Because it takes from 40 minutes to 60 hours for photoinhibition to relax or repair, there is always some residual photoinhibition built into measurements made after a sunny summer day in the field. To measure photoinhibition it may be necessary to limit the amount of light on test plants for about three days before dark adapting to allow complete relaxation or repair of photoinhibion. See the section on dark adapting for more information.

Quenching Parameters

"Flash Count" This is the number of saturation flashes that will occur while the actinic light is on. It ranges from 1 to 99.

"Flash Rate" This is the time between saturation flashes. It ranges from 1 minute to 99 minutes. It has been found that saturation pulse NPQ or the delay in going back to the original chlorophyll fluorescence level after the saturation flash takes up to 2 minutes to dissipate. As a result it is recommended that saturation flashes be at least 2 minutes apart. The total time for the actinic light to be on is based on the flash count multiplied by the flash rate. With the new research regarding chloroplast migration (Cazzaniga 2013) it is probable that this time will be from 20 to 35 minute to ensure proper chloroplast migration and steady state parameter measurement. See the section on chloroplast migration for more information.

"Equations" The choices here are **"Kramer"** lake model protocol parameters that use FO' in their calculations and include q_L, Hendrickson –Klughammer (or **"Klughm"**) lake model protocol parameters that do not use F_O' in their calculation, but do include NPQ, resurrected from the puddle model, and **"Puddle"** model protocol parameters. Tap the button to select the protocol of interest. For more information see the section on quenching overview.

"Actinic" This parameter is used to set the actinic light intensity. Actinic light is the light used to drive photosynthesis. OSI uses a white light LED light source with an intense blue spectrum that ensures chloroplast migration at higher light levels. The intensity may be set from 0 to 1,800 µmols, brighter that any sun light on earth. The intensity is held constant by using the PAR clip. The PAR sensor measures the actinic light every second and makes

adjustments accordingly. This ensures that the actinic light intensity at the leaf will remain the same throughout the quenching test.

Relaxation Parameters

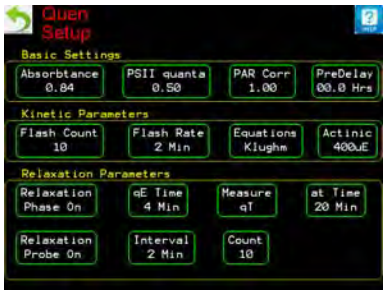
"Relaxation Phase On" To measure q_E, q_M, q_Z, q_T, or q_I, turn the relaxation phase on. If not, turn the relaxation phase off by tapping this button. For information about these parameters see the quenching overview.

"q_E Time" When the relaxation phase is on, q_E or the photoprotective mechanism that it represents can be set. The literature states that q_E ranges between 2 to 7 minutes depending on plant species and growing conditions. Field plants usually take longer. See the quenching overview for more information about q_E (Baker 2008)

"Measure q_M" This is an intermediate relaxation phase. By tapping the button, one can scroll through q_T, q_M, or q_Z. Measuring screens and the data file will report the value type selected. q_I uses the this intermediate value and F_M for determination (Cazzaniga 2013).

"at Time" This is the time that q_T, q_M, or q_Z will be measured. It is likely that survey test should be done to allow time selections for these parameters. According to the literature, q_T takes between 15 to 20 minutes, q_M takes between 20 to 35 minutes and q_Z takes between 15 to 30 minutes. For more information on these parameters see the quenching overview.

"Relaxation Probe On" This refers to the use of saturation flashes at a pre programmed distance apart. It is recommended for survey relaxation measurement, or if one just wants to see the change in relaxation over time. If it is turned off, then the only relaxation saturation flashes that occur during the "relaxation phase on" mode will be at the selected times for q_E and either q_T, q_M or q_Z.



"Interval" This is the time between saturation flashes when the "Relaxation Probe On" is used. It takes about 2 minutes for saturation pulse NPQ to fully dissipate according to the literature. As a result saturation flashes should be at least 2 minutes apart to prevent a build up this type of NPQ. Make sure that this distance corresponds to the qE and qM (or other intermediate saturation flashes) or an additional flash will occur without proper spacing and there will be a build up of saturation pulse NPQ. See the section on quenching overview for more details.

"Count" This is the number of saturation flashes that will occur if the "Relaxation Probe On" feature is used. The probe flashes will end after the number of flashes selected occur. If the number of flashes selected does not reach the intermediate relaxation time, a flash will still occur at the intermediate relaxation flash time selected.

Quenching setup Page 2

Modulation Light

Modulated light intensity may be set automatically or manually. The Intensity and Gain buttons are used to set it manually and the Auto Setup button is used for automated setup.

"Intensity" Modulated light is the measuring light that turns on and off with a specific frequency that is determined at the factory for optimal measurement. The frequency can be different for different measuring protocols. This intensity is very low. When set correctly this intensity is high enough to allow measurement of minimum fluorescence F_O without photochemically reducing any Q_A. If it is set too high an error will occur because some Q_A will be reduced and F_O will be raised. This intensity button, along with the sensor "Gain" button allow manual adjustment of the modulated light intensity. It should be set high enough to allow a measurement, but low enough so that the Ft or fluorescence signal does not start to rise over 10 to 15 seconds while a leaf is in the measuring cuvette. The intensity may be set from 0 to 100%. At 100 %, the intensity is less than 1 μmol.

"Gain" This is the fluorometer photodiode gain. It allows manual set up of modulated light intensity. In general electronic noise increases as the gain increases.

"Wavelength" the OS5p+ offers both a red and a blue modulated measuring light. The default setting is red. For most applications red is used; however the blue may be used for specialty application as well as cyanobacteria and some types of algae. Tap this button to change the color.

"Auto Setup" This allows automatic modulated light adjustment to leaf samples. Place a representative leaf to be measured into a measuring cuvette, and tap the Auto Setup button.

Saturation light intensity

"Intensity" The saturation flash intensity is adjusted by this button. It ranges from 0-100%. At 100% it represents 15,000 μmols. The saturation flash is a white light LED. According to the literature, intense saturation flashes do not harm light adapted samples; however, if they are too frequent, they can harm samples in the dark. This adjustment covers all phases of a quenching protocol. The saturation flash is designed to photochemically reduce all available PSII reaction centers, or close them. In dark adapted samples this works. Results also

correlate to gas exchange measurements in light adapted samples except at high actinic light levels. Here, it is believed that all reaction centers can not closed even with the most intense

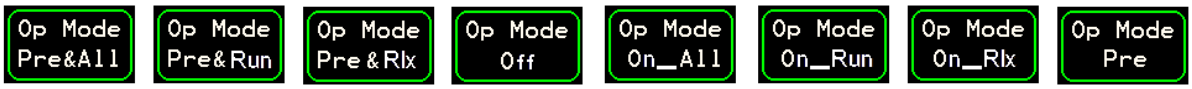
saturation light. For this reason, Loriaux 2006, and more recently, Loriaux 2013 have found an accepted solution that allows estimation of the saturation flash F_M', or light adapted F_M, if as an infinitely intense saturation flash were used. The square topped saturation flash is the default method; however the Loriaux 2013 F_M' correction can be selected using the flash button. Results show that values are the same as the square flash values on dark adapted samples and on samples that are light adapted to low and medium light levels. At higher light levels the difference increases. (For more on this topic see the section on MultiFlash)

"Flash Width" This may be set from 0.4 to 2.0 seconds. The optimal width varies by species. Land plant optimal settings vary from 0.5 seconds to 1.5 seconds. Algae vary from 25 ms. to 50 ms. Opti-Sciences includes a special algorithm that eliminates this as a source of error as long as the saturation flash is wide enough. It uses a 25 ms. 8 point rolling detection average to determine the highest F_M, or F_M' regardless of saturation pulse NPQ. Therefore, it can be used for algae work without concern. For this reason, the default value is set at 0.8.
"Flash" The choices here are the standard square top flash listed as **Square**, and **MultiLvl.** The multiLvl is something we call Mutiflash, or a multiple-phased single saturation flash according to the research of Loriaux 2006 and Loriaux 2013. The protocol follows the latest recommended Loriaux 2013 protocol. The Initial flash is 7,000 µmols for 0.3 seconds, the down ramp is 20%, the down ramping rate is less than 0.01 mol photons $m^{-2}s^{-2}$, and then the final intensity flash is at 7,000 µmols again for 0.3 seconds, to check for saturation flash NPQ (Loriaux 2013). It has been found that under near saturating light conditions that even the most intense saturation flash will not close all or the available reaction centers, causing an error. The Loriaux 2013 method corrects for this error by using least squares linear regression to determine Y(II) and ETR with an infinitely intense saturation flash. This is not an issue at lower light levels. The Multiflash may be used in the Y(II) protocol or in all quenching protocols if it is selected.

Far red light

"Intensity" Far red light is used in the quenching protocol for various reasons. Far red light stimulates PSI, and it is found in sun light so there is no reason to use it for field work in the sun. Far red light is intentionally filtered out of the white actinic light source to allow more control over measurement. One should consider using the far red light when pre-illuminating samples using the built in stable white actinic light source. Set the intensity to 100%.

"Duration" The duration may be set from 1 to 15 seconds for pre-illumination, for Kramer protocol F_O' measurements that are made, and for puddle model q_N and q_P values. The literature shows that it is common to use a value of 5 seconds or 10 seconds, with lower Fo' results at 10 seconds. Under **"Op Mode"** far red light may be turned on or off during the entire quenching protocol test, or just for specific sections of a test. If it is turned on for the entire test F_O' is still determined in the Kramer and puddle model quenching protocols. If it turned off, Far red light only occurs at F_O' determination for the programmed time.
It can also be selected for additional sections of the protocol, like Pre or before F_O and F_M are measured.



"Op Mode" This may be set to allow far red light to be turned off or on during the entire quenching test, turned off during the entire quenching test, or during specific sections of the

quenching test. The default setting is "Off". If Kramer or puddle model protocols are chosen Far red light will still be used for F_O' measurement.

"Pre&All" Far red light will be on before F_O and F_M are measured, and during the entire quenching measurement protocol including relaxation.

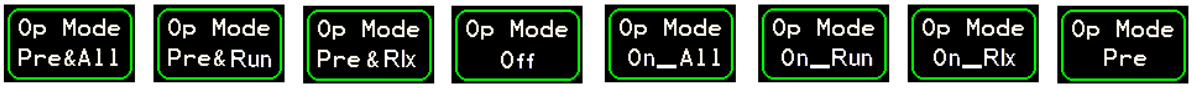
"Pre&Run" Far red light will be on before F_O and F_M are measured and during light adapted portion of the quenching protocol.

"Pre&Rlx" Far red light will be on before F_O and F_M are measured and during dark adapted quenching relaxation portion of the quenching protocol only. It will also be on for F_O' determination in Kramer and puddle protocols.

"Off" Far red light is off except when F_O' is measured during the Kramer lake model quenching protocol, and the puddle model quenching protocol. (This is the factory default setting.

"On_All" Far red light will be on during the entire quenching measurement protocol including relaxation, but not before F_O and F_M are measured.

"On_Run" Far red light will be on during the entire quenching measurement protocol excluding relaxation, but not before F_O and F_M are measured.



"On_Rlx" Far red light will be on during relaxation, but not before F_O and F_M are measured and not during the other phases. It will also be on for F_O' determination in Kramer and puddle protocols.

"Pre" Far red light will be on before F_O and F_M are measured only. It will also be on for F_O' determination in Kramer and puddle protocols.

"Preillumination Time" This button is used to set the time duration of pre-illumination with red light. It may be set from 1 to 60 seconds. If pre-illumination is used, it is common to use times of 5 or 10 seconds.

Data Management

"Log File Name" If this button is tapped a screen will appear that offers **"New"**, **"Existing"**, or **"Cancel"** options. If New is selected, then a screen with "New", **"Kwik Name",** or "Cancel" can be selected. If "New" is tapped on this screen, an alpha numeric keyboard will appear and a 8 character name may be input. Tap the return key to save the name. If "Kwik Name" is tapped, today's date and time become the new file name. If "Cancel" is tapped, the instrument goes back to the previous screen. If "Existing" is tapped, a list of existing files with previously set parameters appears. By tapping one of these file names, the file and the parameters that were created earlier will be loaded into the active memory and used for measurement.

"Add a Note" To add a note for a specific measurement, tap this screen. An alpha numeric keyboard will appear and a 34 character note may be entered into the data file. Tap the return key to save the note.

Once the measuring parameters are set, save the selected parameter as a test by tapping “Save Presets” (See below).

“Load Presets” and **“Save Presets”** The OS5p+ provides two types of files, preset files and data files. Measuring parameters, once set, are stored as preset files by tapping “Save Presets”. To recall saved Preset files, tap “Load Presets”. Data files are created, recalled, and appended when the quenching protocol is opened or under **Data Management “Log file Name”** If Measuring parameters are changed while using a specific data file, the new parameters are listed above the data in the data file, collected after the change, automatically. When saving a set of measuring parameters to a preset file, tap the “Save Preset” button and the options **“New”**, **“Existing”** or **“Cancel”** will appear.

If “New” is selected, an alpha-numeric keyboard will appear and allow an 8 character name to be input. When finished, tap the return key to save the file. The suffix for Preset files varies with the measuring protocol, the suffix for data file names is .CSV .

If “Existing” is selected, a previously made list of Preset files will appear. Select a file to be modified, or tap the “X” on the top right hand side of the orange box to cancel. If one has important Presets files or data files, copies should be saved to a data card for future retrieval. This way, if multiple researchers are using an instrument, the important measuring parameters and data can be saved safely.

After the Presets have been saved, the test is almost ready.

Tap the return arrow on the upper left hand corner of the screen and go back to the main measuring screen. Survey the parameters shown in the upper left hand window to make sure that they are set as needed. Tap this window and the second and final window of parameters will appear. Check this window as well to make sure that these parameters are correct.

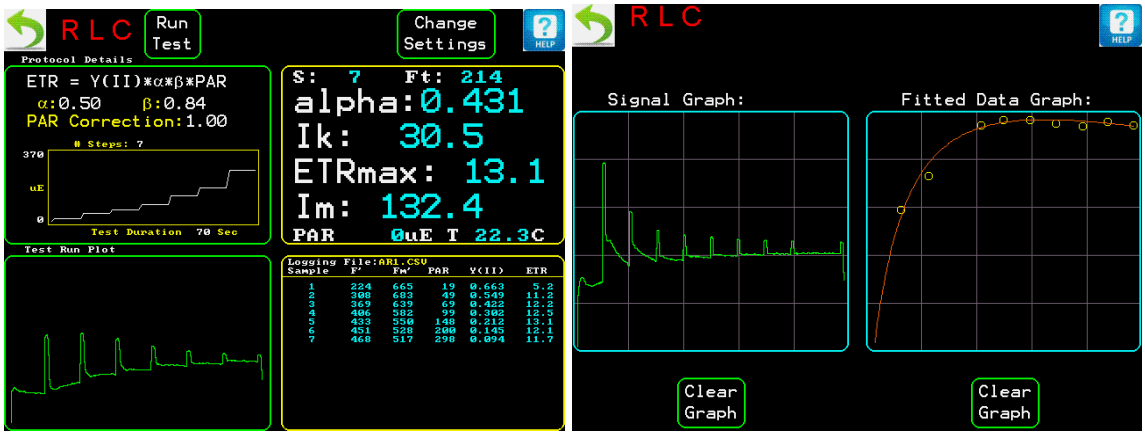
Review the quenching overview in this document for suggested settings and issues that are important.

See the section dark adaptation for recommendations regarding the various mechanisms that affect dark adaptation.

It is recommended that the PAR Clip be used with a camera tripod to make quenching measurements. This ensures that the internal actinic white light source is always at the correct intensity. We recommend a dark or black hand towel or similar covering for dark adaption, or a darkened room. Pre-dawn dark adaptation is also recommended.

With the new evidence regarding chloroplast migration and its affects on chlorophyll fluorescence NPQ it is recommended that at high actinic light levels that light adapted times account for this issue. If it has not been investigated, then 35 minutes is safe (Cazzaniga 2013). Also, it takes from 20 to 35 minutes for chloroplast migration to relax in the dark.

Rapid Light Curves an overview:



Eilers and Peeters 1988 curve fitting and caluculated parameters

Rapid light curves have been heavily used by researchers to study aquatic plants, and for under canopy research on land. Most traditional fluorescence parameters and methods run into difficulty when the light level changes rapidly, or when one is studying the effects of sun flecks. While the measurement of quantum yield of PSII only takes a couple of seconds, it is defined as a measurement taken during steady state photosynthesis, a process that takes between fifteen and twenty minutes at given light level (Maxwell and Johnson 2000) or under high light levels 20 to 35 minutes (Cazzaniga 2013) due to chloroplast migration. Under canopy leaves can be exposed to sun flecks and variable illumination. Traditional quantum yield of PSII, ETR, and standard light response curves run into problems. Using methods that are designed for steady state measurement creates measuring errors when used under changing light conditions. Wave action, changes in water column depth, tides, currents, clouds and turbidity constantly change the amount of light that is received by aquatic plants. Of course, F_V/F_M may be used to measure the health of PSII with some of these situations; however, the need to study and measure the reaction of plants under changing ambient conditions and the need to study light saturation characteristics and plant productivity has driven research into methods such as rapid light curves.

Rapid light curves provide relevant information on the saturation characteristics of electron transport. (Schreiber 2004). When working with Aquatic plants, and water column productivity, saturation characteristics are among the most important determinates. Marra (1978), (Banse and Yong 1990).

Light saturation rate, as measured by rapid light curves, highly correlates with the concentration and maximum activity of Rubico (Macintyre 1997), (Macintyre 1996). Measured Steady state photosynthetic rates overestimate actual photosynthetic rates in a variable light environment (Macintyre 1997).

There are many claims about the value of rapid light curves (or RLCs), and some are controversial. It has been mentioned by Ralph (2005) that a possible reason for rapid light curves not being more highly used is that researchers are not sure what the data means, and how reliable the data is.

We will provide an overview of the rapid light curve, and the research regarding RLC.

How they work:

Rapid light curves are created by dark adapting samples for a specific period of time and stepping a photosynthesis driving actinic light source for short specific periods of time at specific intensities. The light source is usually built into the fluorometer and the sample is shrouded to allow only the actinic light from the fluorometer to hit the sample. Steps may be up or down. Typically, after a short period of time at a specific actinic light level, a single saturation pulse is triggered and the internal light source steps to the next pre-programmed light level.

The yield and ETR values for each step are reported on a separate screen and in the measuring file for file transfer, graphing, and curve fitting software. Relative ETR is calculated using a quantum yield of PSII measurement taken at a given light level and using the equation: rETR = (Quantum yield of PSII) (PAR or the light level) (0.84) (0.5). rETR is scaled on the Y axis, and PAR (photosynthetically active radiation per meter squared per second) is on the X axis.

It is common for the first measurement to be made in the dark and the second step at a low PAR level. It is also common for successive steps to be measured at higher light levels with the last two steps to being measured at or above the leaf light saturation Intensity values are commonly equally spaced. The OS5p+ uses Eilers and Peeters curve model curve fitting software resident on the instrument. It was chosen because it was the most widely used. If other curve fitting models are wanted, the data file can be used with sigma plot software. The data can be put into Sigmaplot software where a regression wizard can use a curve fitting model of choice to determine Cardinal points for analysis. (Ralph 2005)

Web address for Sigmaplot software:

http://www.sigmaplot.com/products/sigmaplot/sigmaplot-details.php .

Cardinal points are then derived from equations determined by the curve model of the researcher's choice. Jassby and Platt (1976), Platt (1980) and Eilers and Peeters (1988) have been used.

α is the initial slope of line at low PAR values created by relating ETR to PAR. It provides a measure of quantum efficiency (Schreiber 2004)

ETR_{max} or p_m is a measure of a leaf's photosynthetic capacity or maximum electron transport rate (Schreiber 2004).

I_k is the Eilers and Peeters calculated point where light saturation dominates, or the minimum saturation level (Schreiber 2004). (Ik is also called Ek in some literature (Ralph 2005))

I_m is the Eilers and Peeters calculated optical actinic light intensity to reach ETR_{MAX} or p_m.

According to Ralph (2005), the initial slope of the curve, α, is proportional to efficiency of light capture.

While photochemical quenching predominates before Ik, non-photochemical quenching dominates after Ik. After the RLC reaches the peak value, any decline in ETR is related to down regulation and not photo-inhibition (Ralph 2005).

The first measurement is taken with no actinic illumination and either dark adaptation or "quasi-dark adaptation". While the number of steps, the intensity of each step, the length of time for actinic illumination at each step, and whether one steps up or down can be variables in RLCs. Some fluorometers offer only a programmed routine that limit choices.

The second measurement is usually low in the 10 to 50 µmol range, and the last two steps are usually above saturation levels. Ralph (2005) recommends that saturation should be at or above 1000 µmols. Low light leaves require a different intensity range for measurement than high light leaves. Intensities above 300 µmols will commonly saturate low light leaves.

The length of actinic illumination is also programmable in some fluorometers. Times of 10 seconds, 20 seconds 30 seconds, 40 seconds, 50 seconds and 60 seconds have been used. Ralph (2005) indicates that Rapid light curves should provide the shortest actinic light time possible to prevent plants from reaching steady-state or causing photo-acclimation. The minimum length for actinic time at each step is governed by the time it takes for the saturation pulse fluorescence signal to relax according to Ralph (2005). High light leaves tend to relax faster than low light leaves according to Ralph. Ralph finds that 10 seconds works for the samples he has measured.

Ralph also shows that all RLC cardinal points, including ETRmax , change substantially with different actinic step times.

Since RLC vary significantly at different times of day because of different light history (Rascher 2000). The data from several light curves, taken at different times of day, can be added together and then subjected to light curve fitting software (Rascher 2000).

What are the limitations of RLC?

The rapid light curve is affected by immediate light history and longer term light history (Rascher 2000), (Ralph 2005). It is also affected by time required to dark adapt (Rascher 2000), NPQ related to the previous light step (Herlory 2007), and in many cases the NPQ from the previous saturation pulse (Roseqvist and van Kooten 2005). These factors will be discussed below.

Rascher (2000) explored the value and limitations of Rapid Light Curves in detail and found that not only did the results from RLC and ETRmax change dramatically depending on the time of day that they were measured, but that they also provided different α and I_k information with different dark-adaptation times. The slopes were found to be steeper with 30-second dark adaption than with 30-minute dark adaption. ETRmax was found to be the same with both dark adaption times. Since light history changes the results of RLCs, Rascher recommends making measurements at different times of day, combining the data, and then feeding it into light curve fitting software.

Ralph (2005) recommends a 5-10 second dark adaption time to measure the effects with no actinic light to drive photosynthesis. This allows for rapid reoxidation of Q_A without significant relaxation of non-photochemical quenching. Ralph also recommends the 5-10

second dark adaptation to prevent the deactivation of rubisco and to prevent a rubisco reactivation induction effect.

Related information in this area includes the following: Full rubisco deactivation takes between 9 to 18 minutes in Algae and up to 28 minutes in land plants (MacIntyre 1997). Full reactivation of Rubisco takes between 3 to 4 minutes for both algae and land plants (MacIntyre 1997). Consalvey (2004) found that far red light illumination used to activate PSI was very helpful in the complete reoxidizing Q_A in a short period of time, whereas dark adaptation took much longer on his samples.

Herlory (2007) found that the time of actinic illumination impacts results. Each successive RLC step adds non-photochemical quenching to the next step. He also found that the time used for actinic illumination at each step affects the repeatability of the results. The most repeatable results were achieved with actinic step times of 50 seconds or longer, and the lowest precision was found with 10 second times.

Roseqvist and van Kooten (2005) found that saturation pulses create a short lived NPQ that takes between 60 seconds and 120 seconds to fully dissipate, so if the actinic steps are shorter than that time frame, then each saturation pulse in the RLC will reduce the yield and ETR values as well.

Conclusion:

Even with these limitations, the literature supports that Rapid Light Curves offer a tool to investigate the saturation characteristics of plants. One must be careful in drawing additional conclusions. In healthy plants the saturation characteristics are about double the values at steady state photosynthesis according to Rascher (2000).

Cookbook check list for rapid light curves

Since light intensity must be stepped up or down in known and stable increments, some advanced Chlorophyll fluorometers allow the uses of an internal actinic light source to create these curves. Other manufacturers recommend the use of external light sources only, for some models of fluorometers. The reason for the concern is that most internal fluorometer light source intensities will change significantly in a short period of time as the fluorometer heats up. The light source itself is the source of the heat. The warmer the fluorometer becomes, the more the light source intensity is reduced. One manufacturer states that their internal actinic illuminator should only be used for less than two minutes, and that light intensities may change by as much as 10% to 20%.

When using the OS5p+, the stable white light LED light source is designed to provide a stable white intensity over time using the PAR Clip to ensure light intensity stability. Tests have shown that the intensity of the LED actinic light source changes less that +/- 3 µmols over an extended periods of time. Rapid light curves take between 1.67 minutes to ten minutes to complete depending on the actinic time used for each step.

The protocol variations available for making rapid light curves are significant, as a result, we will provide general information here, with a specific focus on the Ralph 2005 method. Dark

adaptation, actinic step length, stepping light up or stepping down, and light history issues have been dealt with by different researchers in different ways.

Rapid light curves have been valuable for variable light environmental testing

1. Y(II) requires steady state conditions for reliable results. RLCs, on the other hand, are designed to measure saturation characteristics of plants in a variable light environment. Commonly, actinic step times are designed to measure PSII before the plant has had time to react and adapt to new light levels. Ralph (2005) uses ten seconds, however, other times, up to one minute have been used. Ralph says that dissipation of saturation pulse NPQ governs the minimum time between light steps and saturation pulses, however, complete dissipation may take between one and two minutes. (Roseqvist and van Kooten 2006). As a result, there may be a small amount of saturation pulse NPQ added with each step.

2. Dark adaptation. Ralph recommends a momentary dark adaption of five to ten seconds, using far red pre-illumination to fully oxidize PSII. Other times have also been used (Rascher 2000).

3. RLC Y(II) values vary with light level and with temperature. The higher the light level, the lower the Y(II) value. When measuring Y(II) in the field, it is extremely important to measure leaf irradiation or light level at the leaf and leaf temperature. Comparing Y(II) values taken at different light levels and different heat levels introduces a significant error unless it is the change at different light levels and heat levels that is of interest. This is commonly done with a PAR Clip. If possible, using a PAR clip with a shroud is the recommended approach. This allows the measurement of PAR irradiation level instead of estimates.

4. Shade leaves vs. Sun leaves. – ETR_{MAX} will be higher on sun leaves than on shade leaves Sun leaves should saturate at about 1000 µmols and shade leaves at much lower intensities, 100 to 300 µmols. (Ralph 2005)

5. Field plants should only be compared to field plants and green house plants should be compared to green houseplants due to light history. (Lichtenthaler 2004)

6. Leaf orientation. Is not important

7. It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants (Reuter and Robinson 1997)

8. The duration of the saturation pulse should be between 0.5 seconds and 1.5 seconds for higher plants, and 25 to 50 milliseconds for Phytoplankton and cyanobacteria. Times outside these ranges increase the error in Y(II) measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity (Roseqvist & van Kooten 2006). Longer durations create a form of saturation pulse NPQ that rounds the tail end of the pulse maximum value, and reduces the average maximum saturation pulse value (Roseqvist & van Kooten 2006). Some fluorometers allow adjustment of this parameter, and others are preset at the factory at either. 0.8 seconds, or 1.0 seconds for higher plants. 0.8 seconds is the default value on the OS5p+ and it will work well with almost all higher plants. The OS5p+ has a special algorithm that ignores the effects of saturation pulse NPQ on Fm', for algae, cyanobacteria, and higher plants. As long as the duration is long enough, the correct measurement will be made. Due to short actinic step times, some residual saturation pulse NPQ may be included in the Fs value of the next measurement.

9. Saturation pulse intensity. Saturation pulse intensity is more of an issue with Y(II) than with Fv/Fm. When dark adapting, shade leaves will saturate at a few hundred μmols, and sun leaves will usually saturate below 1,500μmols. However, a problem has been found when measuring Y(II) at high light levels. It has been discovered that at high actinic or sun light levels, leaves resist the complete closure of all PSII reaction centers that is expected when using a saturation pulse. Even with a 7,000 μmol saturation pulse, some reaction centers remain open. Up to a 41% error was found in Y(II) measurements using standard techniques at high actinic light levels. To correct for this issue, multiple saturation flashes are used, and the measured maximum fluorescence value for each flash is entered into a linear regression analysis formula to determine the maximum fluorescence intensity with an infinite saturation flash. The multiple saturation pulse approach has been shown to work in multiple papers and posters. It may be used with RLCs, However, there we are not aware of research where this technique is used for RLC. To date the research has only been done on leaves at steady state photosynthesis. The method follows the latest method and settings described in Loriaux 2013. The resulting value has been shown to correlate well with gas exchange carbon assimilation values. This multi-flash method is available on the OS5p+ and OS1p fluorometers. (see the Multi-flash section for more details).

10. PSI fluorescence - Part of the fluorescence signal contains PSI fluorescence as well as PSII fluorescence. With Y(II), one is trying to measure variable fluorescence of PSII in a light adapted state.

PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces an a small error. This is not a problem for comparing similar samples, because PSI fluorescence does not change with light intensity temperature or plant stress.

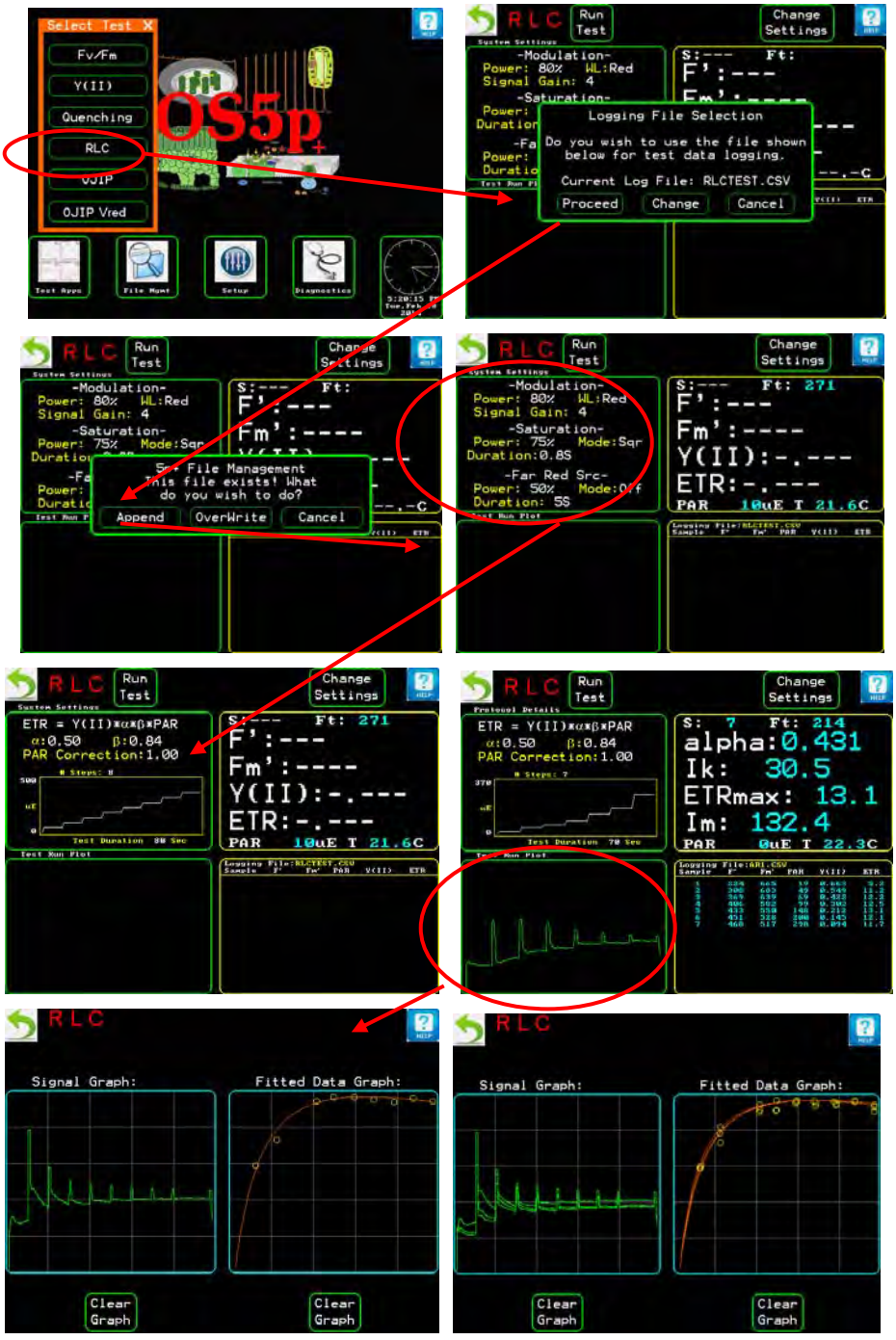
11. Samples with different light history may contain different amounts of photoinhibition. Lichenthaler (2004) found that photoinhibition starts to relax at 40 minutes and it can take from 30 to 60 hours for complete relaxation or repair of photoinhibition. For this reason, it is important only to compare samples with similar light histories. Lab samples and green house samples may be shaded for three day to remove chronic photoinhibition as a variable.

12. Heterogeneous fluorescence. Fluorescence becomes patchy in plant leaves that are under drought stress, cold stress, or at low CO_2 levels. Under these conditions, on larger leaves, it may be best to plan three of four different measurements per leaf and come up with an average. (Buschmann 2004, and correspondence with Buschmann).

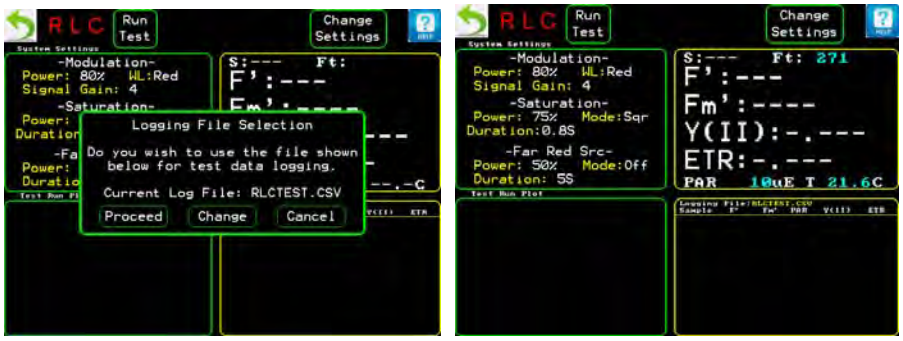
PAR is photosynthetically active radiation. Radiation on the leaf is measured between the wavelengths of 400nm to 700nm. PAR sensors and thermistors for measuring temperature are calibrated to other instruments that are traceable to the NIST. It is recommended that recalibration should occur every two years. Most modern sensors are solid state, so drift is minimal.

Running the RLC (Rapid light curve) test

From the main screen select **"Test Apps"** by pressing the touch screen. The Test Selector screen will appear. Tap the **"RLC"** button.



RLC Screens

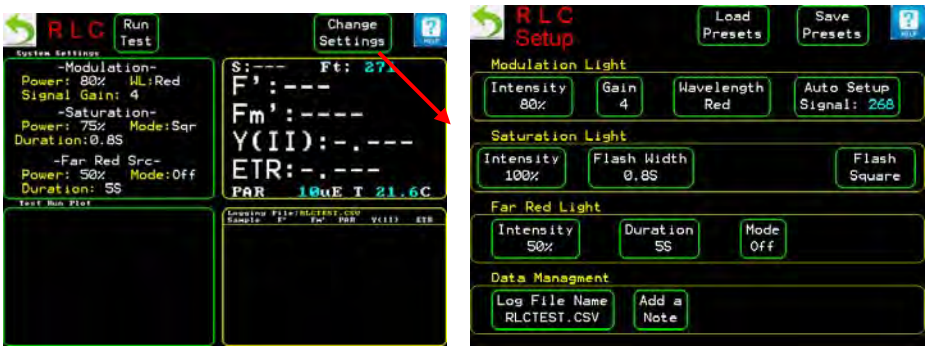


Next, one can choose to proceed with the last measuring file and set of instrument settings that were used by tapping **"Proceed"**. The existing file can also be changed and renamed later. If **"Change"** is tapped it, is possible to select another measuring file, create a new one, or the process may be canceled by tapping **"Cancel"**. If cancel it pressed, the instrument goes back to the previous screen.

When **"Proceed"** is pressed, there are three choices. **"Append"**: Add measurements to the existing file. **"Cancel"**: Go back to the main menu; and **"OverWrite"**: Use the file that is currently being used but the new measurement will overwrite the previous measurement.

To measure, tap **"Run Test"** on the measuring screen.

On the RLC measuring screen, a green square means that more information can be seen by touching that green window. The **"Change Settings"** button allows the settings displayed in the upper left hand corner window to be changed. For other parameters, tap the window in the upper left hand corner first.



When the **Modulation** topic is in the upper left hand corner window, the setup screen shown above appears. **"Load Presets"** allows previously created preset files to be opened and used. **"Save Presets"** allows the current set up parameters to be saved in a Preset file. Measuring parameters are saved in Preset files and data is stored in a separate data file. The presets used during measurement appear above the data. If presets are changed, the new presets will appear above the data that follows it, and after the data collected using different presets.

The section under Modulated Light is for adjustment of the measuring light. This light turns on and off rapidly. The frequency for each test is set at the factory to ensure reliable operation automatically.

The OS5p+ provides a red modulated light source and a blue modulated light source. Select the type of light source that you want to use under **"Wavelength"**. Red is the most used light source. Blue may be used for cyano-bacteria some algae and for other application. If you do not know which light source to use, it is likely that red is the best choice.

For RLCs, the **"Intensity"** must be high enough to measure a fluorescence signal but not too high. If it is too high it will become actinic and cause a partial reduction of Q_A. If this happens, an error will result that reduces saturation flash. The correct setting will allow a fluorescence signal measurement without the reduction of any Q_A.

The OS5p+ has an automated routine that ensures that the modulated light intensity and the gain are set just right. Place a dark clip on the type of leaf that will be used. Insert the fiber optic into the opening of the dark clip, and then open the dark slide. The leaf will be exposed to the existing modulated light level. Next tap the **"Auto Setup button"** in the set up screen. Beeps will sound while it automatically adjusts the light source so that the intensity and gain are optimal. This is done to help ensure error free operation.

The **Saturation Light** section is used to adjust the saturation light. The intensity can be set by tapping the "intensity" button. 100% represents 15,000 µmols of irradiation.

The **"Flash Width"** or saturation pulse duration may be set between 0.4 seconds and 1.5 seconds. Opti-Sciences has also semi-automated this function to reduce measuring errors. An algorithm is provided, in all of the modulated protocols, that uses an 8 point 25 ms. rolling average to detect the highest measuring points for FM, and FM', this eliminates saturation pulse NPQ from being a problem. This means, that as long as the saturation pulse is wide enough, the optimal answer will be provided regardless of saturation pulse NPQ. The literature states that land plants have an optimal range of 0.5 seconds to 1.5 seconds. For Algae the optimal times are shorter, they range from 25 ms. to 50 ms. Saturation pulse NPQ is a rounding of the tail end of the saturation pulse fluorescence signal that happens if the flash width is too wide. It is also evident in the fluorescence signal as it slopes down to pre saturation pulse level According to Roseqvist and van Kooten 2006, it takes from 60 seconds to 120 seconds for full dissipation of saturation pulse NPQ. As a result, there is probably some residual saturation pulse NPQ included when step times are less than that, and it is likely increased with more steps. For this reason, fewer steps are probably better than more steps.

"Flash" The choice here is either the standard **Square topped flash**, or **MultiLvl** for Multiflash. (See the section on Multiflash for more information.)

Far Red Light

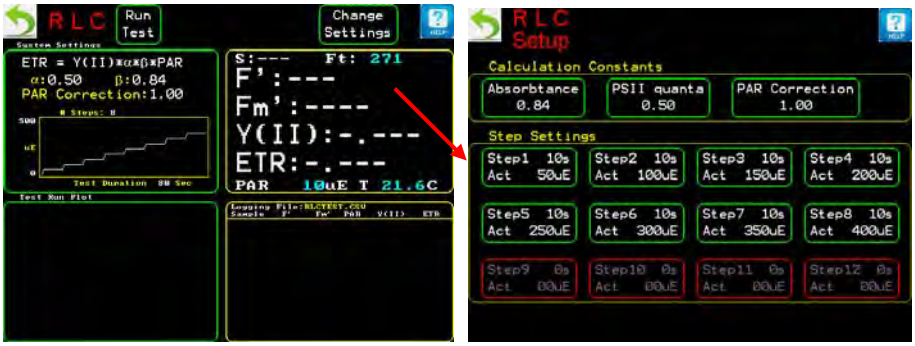
Far red light can be used to ensure complete re-oxidation of PSII by stimulation of PSI. By pre-illumination of the leaf with far red light for 5 to 10 seconds (Maxwell & Johnson 2000). Under far red light, PSI takes the remaining electrons from PSII, ensuring a more fully oxidized PSII. Tests have been done that show that 10 seconds is better than 5 seconds; however, this does add another 5 to 10 seconds to the measuring time. Far red light does not allow the elimination of dark adaptation **(**Consalvey (2004).

In RLC momentary 5 to 10 second dark adaptation is used by some researchers (Ralph 2005), (Rascher 2000). Under these circumstances recent light history is built into all measurements and values will change at different times of day. Other dark adaptation protocols must account for the ΔpH of the thylakoid lumen, the relaxation of xanthophyll cycle, relaxation of chloroplast migration, and state transitions (where they exist), for proper measurement. The **"Intensity"** can be set from 0 to 100%. The **"Duration"** can be set from 1 to 15 seconds. The **"Mode"** button is used for pre-illumination only, off, or on during the entire measurement.

Data Management

"Log File Name" If this button is tapped, a screen will appear that offers **"New"**, **"Existing"**, or **"Cancel"** options. If New is selected, then a screen with "New", **"Kwik Name",** or "Cancel" can be selected. If "New" is tapped on this screen, an alpha numeric keyboard will appear and an 8 character name may be input. Tap the return key to save the name. If "Kwik Name" is tapped, today's date and time become the new file name. If "Cancel" is tapped, the instrument goes back to the previous screen. If "Existing" is tapped, a list of existing files appears. By tapping one of these file names, the file created earlier will be loaded into the active memory and used for measurement. In the standard operating mode (not Multi User mode) a previously developed Preset file must also be loaded to duplicate previous conditions. In the Multi User mode, the instrument will require entering both a data file and a presets file. (See Multi User mode for more details)

"Add a Note" To add a note for a specific measurement, tap this screen. An alpha numeric keyboard will appear and a 34 character note may be entered into the data file. Tap the return key to save the note.



If the ETR = Y(II) x α x β x PAR is at the top of the upper left hand window, then the screen on the right above will appear if that window is on the screen and **"Change Settings"** is tapped.

Calculation Constants

"Absorptance" is an average leaf absorptance in white light. Average plant values are used in the standard equation. 0.84 is a good average value for many species of plant under average conditions (Bjorkman and Demming, 1987), research has shown that the leaf absorption coefficient can vary between 0.7 and 0.9 in healthy plants using a red modulated light source

(Eichelman H. 2004). Absorptance varies with species, chlorophyll content, leaf age, plant stress, and by light level (light level - see Cazzaniga 2013). Note: When using the blue modulated light it was found that the average absorption value should be set closer to 0.94 instead of 0.84. Leaf absorptance is usually measured using an integrated sphere, a light source and a spectrophotometer.

"PSII quanta"

Research has also shown that the fraction of light that is absorbed by PSII reaction centers varies by species, plant type (C_3, C_4 plant or other), sun or shade growing conditions, and under severe carbon deficit. species and can range from at least .40 to .60 (Laisk and Loreto,1996). Some C_4 plants like corn are close to 0.40 and some C_3 plants can be closer to 0.60. While 0.5 is used for an average value, the most used method for measuring the ratio of PSII to PSI, involves the use of spectral analysis of samples at 77^{o}K (Anderson 1999), (Zell 2010);

Baker (2008) states the ETR should not be used for comparing different samples unless leaf absorptance, and the ratio of PSII to PSI reaction centers have been measured. For this reason, Y(II) is more properly used for plant stress measurement.

"PAR Correction"

This is available to adjust for PAR sensor location error if wanted. According to Rascher (2000), there can be up to a 10% error in PAR values due to the difference in location of the sample and the PAR sensor. It is set to 1.00 as a default value.

Step Settings

In this category the number of steps, the duration of a step, and the light intensity at that step may be set. While almost any process may be tried, it is common to start at 20 to 50 µmols and step the RLC to at least one value above saturation. (See the Overview of RLC for more information.)

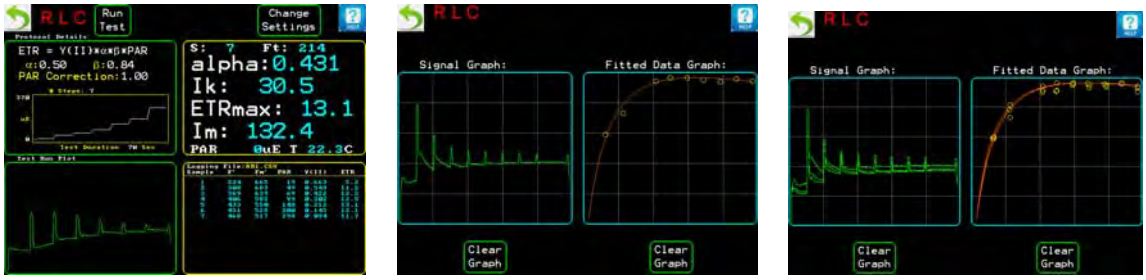
Green boxes are steps that will be used to measure samples and red boxes are disabled.

When a green box step is tapped, the Step **"Dwell"** can be set from 10 seconds to 60 seconds. Actinic Lvl or level may be set from 10 µmols to 1,800 µmols. **"Disable"** disables the step, **"Cancel"** brings the user to the previous screen.

When a red box step is tapped, an orange box appears for Set Dwell time. Input values from 10 seconds to 60 seconds. Tap the return key. Next, another orange box appears to Set Actinic light level. Input values from 10 to 1,800 µmols. Tap the return key when finished. Up to 12 steps may be created. When finished tap the return button in the upper left hand corner to go back to the measuring screen.

This protocol is designed to be used with the PAR clip. It is recommended that the PAR clip be mounted on a tripod of some type. Insert the leaf into the PAR clip chuck, cover the PAR clip with a dark cloth for a specific dark adapted time, and tap run test.

When the test is complete the following screens may be seen. To see the screen on the right, tap the graph screen. Multiple graphs may be overlaid as shown on the right, by running several tests while the specific RLC file is open and the instrument is left on.



The graphic image may be stored by tapping the **"Help"** button and pressing **Capture Screen**. An alpha numeric keyboard will appear and allow an 8 digit file name to be created. The image can be retrieved with the data card placed in the machine and tapping File Mgmt on the main instrument screen. Select the file of interest and tap copy when the screen appears. The file is now on the data card and it may be opened in paint or other programs that accept bit map images.

The Strasser OJIP Protocol: for plant stress measurement.

The Strasser OJIP protocol focuses primarily on plant stress measurement. The OS5P+ is designed to report all of the Strasser measuring parameters in the data file, and provide direct read-out of the most used Strasser protocol parameters including: OKJIP, S, t100, PI_{ABS}, V_J, M_O, time to P, and A. The light source intensity is adjustable from 0 to 5080 µmols. However, the Strasser protocol is commonly run at 3,500 µmols now, and previously at 3,000 µmols. OJIP curves have been found to change with actinic light intensity (Vredenberg 2004, 2011). Parameters reported to the data file include: O at t20 µs, t100 µs, t300 µs (or K), t2ms (or J), t30ms (or I), P, tF_M, A (area above the curve), M_O (or RC/ABS), V_J, PI_{ABS} (or performance index) F_O, F_M, F_V/F_M, F_V/F_O. ABS/RC, TR_O/RC, DI_O/CS, ET_O/RC, TR_O/ABS, ET_O/TR_O, ET_O/CS, RC/CS_O, RC/CS_M, S, M, and T are also measured if the test is long enough.

The OS5p+ OJIP test is a modulated light measured test that actually measures F_O. Continuous fluorometers do not measure F_O but estimate it. The formulas for each parameter measurement are listed in this section.

A discussion of the fluorescence rise and what each step means can be found in the section on variable chlorophyll fluorescence a review 2014. For references regarding plant stress measurement, review the Opti-Sciences Desk Top Plant Stress Guide found on our website: www.optisci.com .

Viewing OJIP graphic results can now be quickly and easily done in the field. The OS5p+ provides a color graphic display of the OJIP curve with a logarithmic time scale and a linear time scale. Up to 32 color traces may be overlaid on the measuring screen. Up to 32 measurement traces can be stored in a single measuring file for later viewing and comparison on your computer. When the file is transferred to Excel or other spread sheets, the first column provides a detailed time stamp for the measurement fluorescence values. The remaining columns, up to 32, provide fluorescence trace values for stored measuring traces.

Originally in OJIP techniques, the overlay of graphs taken from different plants, or the same plant under varying degrees of plant stress, were used to monitor plant stress. Later, various quantitative parameters were developed to provide a more sensitive and more quantitative approach to OJIP plant stress detection. It is still common for researchers to use this overlay technique, to study the effects of plant stress, and to use the special parameters that have been created to detect and quantify plant stress.

Sampling rates and measurement points:

Number of Data Points	Sampling Time Interval	Total Elapsed Time	Parameter Designation
0	0	0	F_O measured
2	10 µs.	20 µs	O - 20 µs
8	10 µs	100 µs	t100 µs
20	10 µs	300 µs	t300 µs K step
27	100 µs	3 ms	t 2 ms J step
27	1ms	30 ms	t 30 ms I step
27	100 ms	3 secs	P = F_M variable time
27	1 sec	30 secs	
27	10 secs	300 secs	

Parameter equations or definitions:

	Direct read out parameters
F_O	Pre-photosynthetic minimum fluorescence measured with weak red modulated light. PSII is oxidized and all available reaction centers are open.
F_M	Maximum fluorescence measured or P. All available reaction centers are reduced or closed.
O	Origin –Fluorescence value at 20 µs.
t_{100}	Fluorescence value at 100 µs.
t_{300} K step	Fluorescence value at 300 µs.
J step	Fluorescence value at 2 ms
I step	Fluorescence value at 30 ms
P Step	Maximum fluorescence or F_M
tF_M	Time to reach P or F_M
A	Area above the curve from t0 to tF_M and from F_O to F_M
Vj	Vj = (Fj – Fo) / Fm-Fo
M_O or RC/ABS	$M_O = (F_{300} – F_{50}) / (F_M – F_{50}) / 0.25ms$
PI_{ABS}	$PI_{ABS} = (Vj / Mo) (F_V/F_M) (F_V/F_O) ((F_M-Fj)/ (Fj-Fo))$
or	$PI_{ABS} = (Vj / (dV/dto)) (F_V/F_M) (F_V/F_O) ((F_M-Fj)/ (Fj-Fo))$
	Parameters recorded in the data file only
S	Value at approximately 1 seconds
M	Value at approximately 3 seconds
T	Value at approximately 30 seconds
ABS/RC	$ABS/RC = (M_O) (1/Vj) (1/(1-(F_O/F_M)))$
TR_O/RC	$TR_O/RC = (M_O) (1-Vj)$
DI_O/CS	$DI_O/CS = (M_O) (1/Vj) (1/(1-(F_O/F_M))) – ((M_O) (1/Vj))$
ET_O/RC	$ET_O/RC = (M_O) (1/Vj) (1-Vj)$
TR_O/ABS	$TR_O/ABS = F_V/F_M$
ET_O/TR_O	$ET_O/TR_O = 1-Vj$

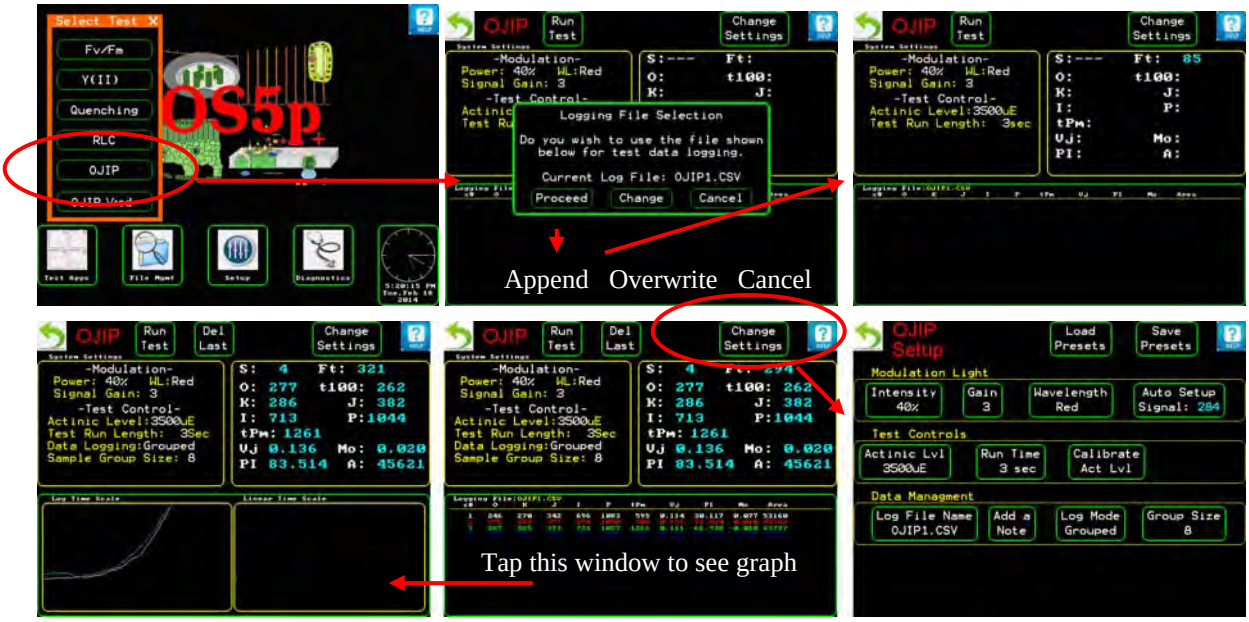
ET_O/CS	$ET_O/CS = (1- (F_O/F_M)) (1-Vj) (F_O)$
RC/CS_O	$RC/CS_O = F_O / (M_O) (1/Vj) (1/(1- (F_O/F_M)))$
RC/CS_M	$RC/CS_M = F_M / (M_O) (1/Vj) (1/(1- (F_O/F_M)))$

Reference :

Strasser R.J, Tsimilli-Michael M., and Srivastava A. (2004) - Analysis of Chlorophyll a Fluorescence Transient. From Chapter 12, "Chlorophyll a Fluorescence a Signature of Photosynthesis", edited by George Papaqeorgiou and Govindjee, published by Springer 2004, PO Box 17, 3300 AA Dordrecht, The Netherlands, page 340-362

Running the Strasser OJIP protocol.

Use the OJIP protocol with dark clips. Do not use the PAR Clip!



From the main screen, tap **Test Apps**, and then **OJIP**. The logging file selection screen appears.

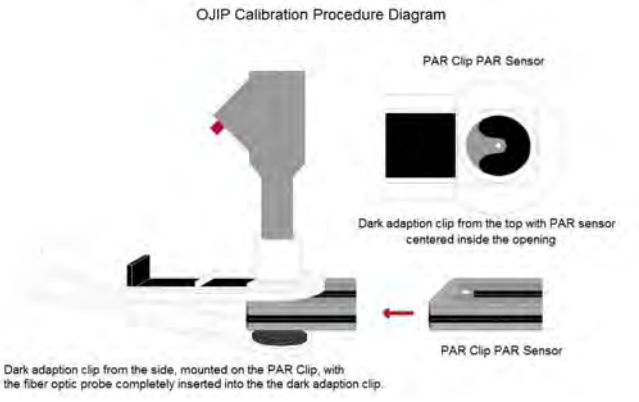
Tap **"Proceed"** and a new screen that lists **"Append", "OverWrite",** and **"Cancel"** appear. Tap **"Append"** and the main OJIP measuring page appears. **"OverWrite"** overwrites the last measurement. **"Cancel"** makes the system go to the previous screen.

Strasser Protocol OJIP Light source calibration

Use the OJIP protocol with dark clips. Do not use the PAR Clip!

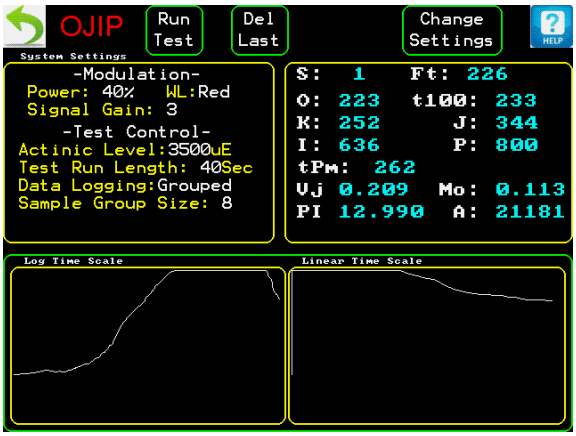
Because OJIP results and the OJIP logarithmic graph change with light intensity (Vredenberg 2004). OSI provides a light calibration routine. While any intensity value may be calibrated, 3,500 µmols is recommended for the Strasser Protocol.

To calibrate the light source, attach the PAR clip to the electronic connector PAR clip port on the side of the OS5p+. Place a dark adaption clip (cuvette), with the dark slide in the open position, on to the PAR clip PAR sensor, with the sensor located approximately in the center of the open dark adaption clip. Insert the fiber optic probe into the dark adaption clip all of the way (See below). Tap **"Change Settings"** while on the OJIP main measuring screen, and the following screen appears:



Tap **"Calibrate Act Lvl"**, when the actinic light level intensity is set to 3,500 µmols. The PAR sensor will read the light irradiation level and adjust the light source intensity until the PAR sensor reads 3,500 µmols. The light source is now calibrated. The calibration can change if the temperature changes significantly. It that case, we recommend recalibration as needed.

Note: Try to hold the dark adaptation clip top, with the fiber optic bundle, perpendicular to the PAR sensor. Perfect alignment is not necessary; however, the diagram below shows the best alignment.



When the OJIP Test protocol measuring screen appears, settings are listed on the upper left hand window. They include modulated light settings **Power**, **WL** for wavelength color, and **Signal Gain**. The OS5p+ has an automated modulated light adjustment to optimally set the correct intensity. It may also be set manually. Power is the intensity of the modulated light. Signal Gain is the electronic gain to the silicon diode sensor. Red modulated light source is recommended for most applications.

Actinic level is the light intensity at the leaf. 3,500 µmols is the default value.

Test Run Length is the time that the test will run. It may be adjusted from 3 seconds to 255 seconds.

Data Logging may be set to **Standard** for single graphing of measurements or **Grouped** for multiple overlay of graphed samples.

Group Size may be set from 2 to 32. This will allow graphic overlay of up to 32 separate OJIP measurements on the log time scale graphic display.

To change these OJIP parameters, tap **"Change Settings"** and the screen below will appear.

"Load Presets" and **"Save Presets"** The OS5p+ provides two types of files, preset files and data files. Measuring parameters, once set, are stored as preset files by tapping **"Save Presets"**. To recall saved Preset files, tap **"Load Presets"**. Data files are created, recalled, and appended when the OJIP protocol is opened or under **Data Management "Log file Name"** If Measuring parameters are changed while using a specific data file, the new parameters are listed above the data in the data file, in data collected after the change, automatically. When saving a set of measuring parameters to a preset file, tap the **"Save Preset"** button and the options **"New"**, **"Existing"** or **"Cancel"** will appear.

If "New" is selected, an alpha-numeric keyboard will appear and allow an 8 character name to be input. When finished, tap the return key to save the file. The suffix for Preset files is related to the measuring protocol used , the suffix for data file names is .CSV .

If "Existing" is selected, a previously made list of Preset files will appear. Select a file to be modified, or tap the "X" on the top right hand side of the orange box to cancel. If one has important Presets files or data files, copies should be saved to a data card for future retrieval. This way, if multiple researchers are using an instrument, the important measuring parameters and data can be saved safely.

To set up the OJIP test, tap the **"Change Settings"** button. The Set up screen will appear.

The section under **Modulated Light** is for adjustment of the measuring light. This light turns on and off rapidly. The frequency for each test is set at the factory to ensure reliable operation automatically.

The OS5p+ provides a red modulated light source and a blue modulated light source. Select the type of light source that you want to use under **"Wavelength"**. Red is the most used light source. Blue may be used for cyano-bacteria some algae and for other application. If you do not know which light source to use, it is likely that red is the best choice.

For OJIP, the **"Intensity"** must be high enough to measure a fluorescence signal but not too high. If it is too high it will become actinic and cause a partial reduction of Q_A. If this

happens, an error will result that reduces P and raises O. The correct setting will allow a fluorescence signal measurement without the reduction of any Q_A.

The OS5p+ has an automated routine that ensures that the modulated light intensity and the gain are set just right. Place a dark clip on the type of leaf that will be used. Insert the fiber optic into the opening of the dark clip, and then open the dark slide. The leaf will be exposed to the existing modulated light level. Next tap the **"Auto Setup button"** in the set up screen. Beeps will sound while it automatically adjusts the light source so that the intensity and gain are optimal. This is done to help ensure error free operation.

The intensity may also be adjusted manually. As before, set the leaf in the dark adaption clip and expose it to the existing modulated signal. If the Signal shown under Auto Setup increased over a 15 to 20 second time frame, the signal is too high and some Q_A is being reduced. Use a different dark adapted leaf and clip. Reduce the intensity until the signal does not rise over a 15 to 20 second time frame. Return to the main measuring screen, and tap **"Run Test".** If the message: "fluorescence signal too low" does not appear, the modulated light is properly adjusted.

Test Controls

"Actinic Lvl" This button is used to set the actinic light level. In OJIP, is customary to use the term actinic light instead of saturation flash. While they do the same thing for the rise in chlorophyll fluorescence, in the OJIP protocol the actinic light can be on for extended periods of time. The actinic level can be set by tapping this button. The range is from 0 to 5,080 µmols. Because results vary with actinic light intensity, the 3,500 µmols setting is recommended for the Strasser protocol. Older systems have used to use 3,000 µmols. It has been shown that leaves grown in low light conditions saturate at about 300 µmols and that leaves grown under high light conditions saturate at about 1,500 µmols. (Ralph P. J. 2005). It is common to use 3,500 µmols for the OJIP test in plant stress testing with the Strasser Protocol; however, Vredenberg (2011) uses various actinic intensities to study the different phases of the OJIP induction curve.

"Run Time" The default setting is for 3 seconds. The time may be adjusted from 3 seconds to 255 seconds. If shorter illumination times are of interest for basic research purposes, use the **OJIP Vred** protocol.

"Calibrate Actinic Lvl" This is used for calibration of the actinic light intensity used in the OJIP protocol. See the section on OJIP calibration for more information. The yellow number to the right of the calibrate actinic level button is the calibration factor after calibration. It will change if the calibration changes. It is a good idea to record this value for future reference.

Data Management

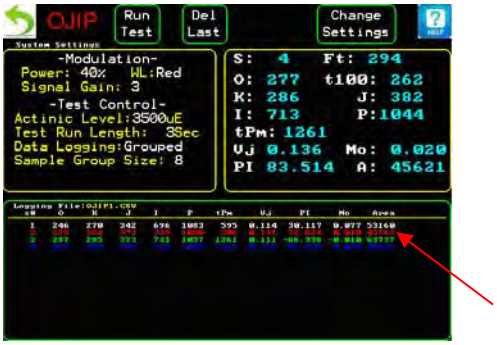
"Log File Name" If this button is tapped a screen will appear that offers **"New"**, **"Existing"**, or **"Cancel"** options. If New is selected, then a screen with "New", **"Kwik Name"**, or "Cancel" can be selected. If "New" is tapped on this screen, an alpha numeric keyboard will appear and an 8 character name may be input. Tap the return key to save the name. If "Kwik Name" is tapped, today's date and time become the new file name. If "Cancel" is tapped, the instrument goes back to the previous screen. If "Existing" is tapped, a list of existing files with previously set parameters appears. By tapping one of these file names, the file and the parameters that were created earlier will be loaded into the active memory and used for measurement.

"Add a Note" To add a note for a specific measurement, tap this screen. An alpha numeric keyboard will appear and a 34 character note may be entered into the data file. Tap the return key to save the note.

"Log Mode" This may be set to **"Standard"** or **"Grouped"**. If Standard is used, a single graphed measuring trace will appear in the graphic displays. If Grouped is selected, then from 2 to 32 measurements may be graphed in an overlaid fashion. There are 32 different colors.

"Group Size" With this button, select from 2 to 32 measuring traces to be grouped together with their fluorescence traces to be overlaid.

Measurement Review

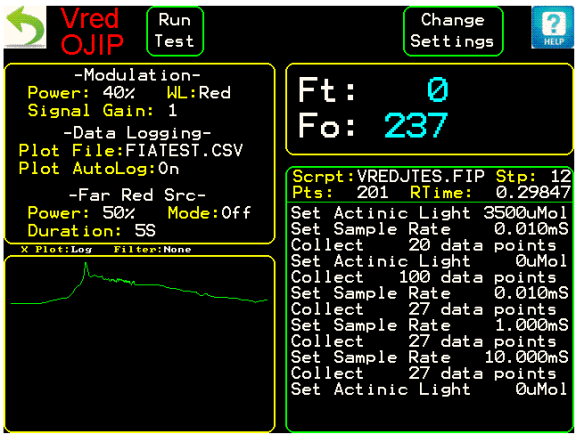


On the main OJIP measuring screen, the lower portion of the screen logs all of the measurements made in the current measuring session for the current data file name. One can scroll through measurements by touching the screen. If one touches the top of the review area, the earliest measurements may be reviewed. If one touches the bottom of the review area, then the latest measurement can be reviewed. The session remains current as long as the file name remains the same and one does not leave the OJIP protocol. It will stay intact if one goes to different screens within the OJIP protocol. If one turns off the instrument, changes the file name, or goes to a different protocol, then the session is ended. All measuring data is retained with the current file name in the measuring data file, and it may be viewed in Excel on a computer. It does not disappear like the measurement review session data.

F_O is measured, not estimated.

Vredenberg OJIP research protocol

This protocol was developed in conjunction with Dr. Wim Vredenberg as a basic research tool to study the fluorescence rise and quenching at high time resolution. It allows changing actinic light intensities, sampling rates, the number of points collected at a specific sampling rate, and the ability to turn the actinic light on or off at any point. It allows a more detailed investigation into such phenomenon as OJIP and nonphotochemical quenching. In the screen shot below, the actinic light was turned on for 0.2 ms and then turned off. The J peak is at about 2.0 ms. Nonphotochemical quenching is still seen after only 0.2 ms. This system uses either a modulated red light or a modulated blue light that allows measurement of pre- Q_A reduction of F_O, and to measure fluorescence after the actinic light has been turned off. FO is measured, not estimated.



It provides a menu driven script writing capability with built in safe guards to prevent script errors. This protocol is not designed for new comers to chlorophyll fluorescence, it is designed for advanced users. The instrument may be purchased with either a Red Actinic light, or a White actinic light. If this protocol is the main focus, Vredenberg recommends a red actinic light source. If longer quenching measurements are the focus, white light is recommended due to chloroplast migration measurement requirements using an intense blue spectrum. See chloroplast migration q_M for more details on this subject. While the size of the measuring file does not have limits, the do not exceed number of points is 65,000. Excel with not work with larger files. Bad things could happen if that number is exceeded. A **purple fluorescent plastic slide** is provided, in a clear case, to measure electronic noise and allow comparison of results to measurements of plant physiology. The slide fluoresces when exposed to light but it does not provide variable fluorescence. As a result, each measuring point can be compared to machine artifacts to verify if results are due to plant physiology or electronic noise.

Vredenberg protocol screen map

To get to the Vredenberg protocol tap Test Apps on the main screen, and an orange window appears. Tap **"OJIP Vred"** to get to the main measuring screen in the middle top row.

Next tap **"Change Settings"** and a second orange window appears. Tap the Edit Settings button and the **Vred Setup** screen appears. The section under Modulated Light is for adjustment of the measuring light. This light turns on and off rapidly. The frequency for each test is set at the factory to ensure reliable operation automatically for each sampling rate.

The OS5p+ provides a red modulated light source and a blue modulated light source. Select the type of light source that you want to use under **"Wavelength"**. Red is the most used light source. Blue may be used for cyano-bacteria some algae and for other application. If you do not know which light source to use, it is likely that red is the best choice.

For OJIP, the **"Intensity"** must be high enough to measure a fluorescence signal but not too high. If it is too high, it will become actinic and cause a partial reduction of QA.

If this happens, an error will result. The correct setting will allow a fluorescence signal measurement without the reduction of any QA.

The OS5p+ has an automated routine that ensures that the modulated light intensity and the gain are set just right. Place a dark clip on the type of leaf that will be used. Insert the fiber optic into the opening of the dark clip, and then open the dark slide. The leaf will be exposed to the existing modulated light level. Next tap the **"Auto Setup button"** in the set up screen.

Beeps will sound while it automatically adjusts the light source so that the intensity and gain are optimal. This is done to help ensure error free operation. It may also be set up manually. Follow the same procedure. If Ft rises over about a 10 to 20 second time frame, when the leaf sample is exposed to the modulated light, then the intensity is set too high. If the signal message comes back too low the intensity and finally the gain should be adjusted accordingly.

The **Test Controls** line allows adjustment of the graphic instrument display of the Vredenberg protocol graph. **Plot X** allows either a Linear display **"Lin"**, or a Logarithmic display of the graph by selecting **"Log"**. The **Post Filter** button allows display of raw fluorescence data with **"No Filter"**, a 3 point average of measured values **"3 point"**, a 5 point average of measured values "**5 point"**, or a 7 point average of measured values with **"7 point"**.

Electronic noise measuring standard - purple piece of plastic

A special fluorescence slide standard is provided to allow comparison of results with chlorophyll fluorescence measurements. The slide provides a non-variable graph that allows viewing and measurement of the electronic noise of the instrument at any point. The noise is very small and most of it is caused by the modulated light being turned on and off very rapidly. This makes measurement position repeatable. By comparing the two graphs, artifacts can be separated from plant physiology. The slide is a **purple piece of plastic** in a clear case. It should be place in the dark clip and tested like any other sample. While signal averaging can be used in its place, it is likely that some will want to see the noise and everything.

Data Management

The **Data Management** allows naming of files, adding notes to measurements, and it allows saving of OJIP fluorescence trace data to the OJIP Vred data file. File naming and notes are done the same way as in other measuring protocols. See those sections for directions.

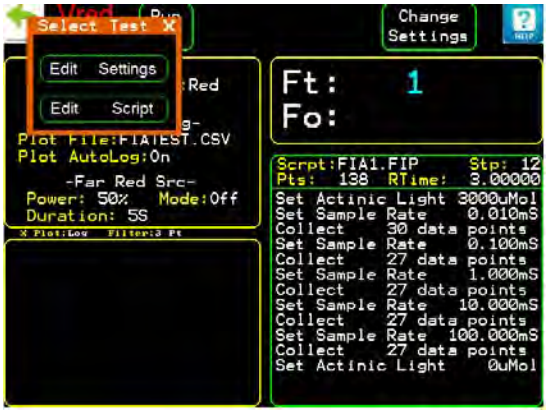
The "**Trace Log"** button is used to save the OJIP fluorescent measuring trace to the data file for analysis in Excel or other comma delineated programs, by selecting **"On"** or to turn this function **"Off"**.

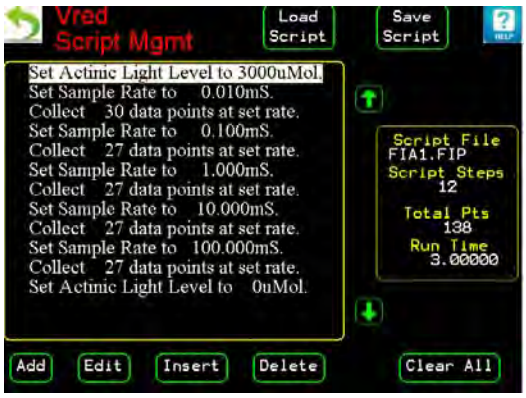
Far Red Light before the trace may be turned off or on. Select **"Mode" "On"** or **"Off"**. The intensity of the Far Red Light before the actinic light is turned on may be set from 0 to 100%.

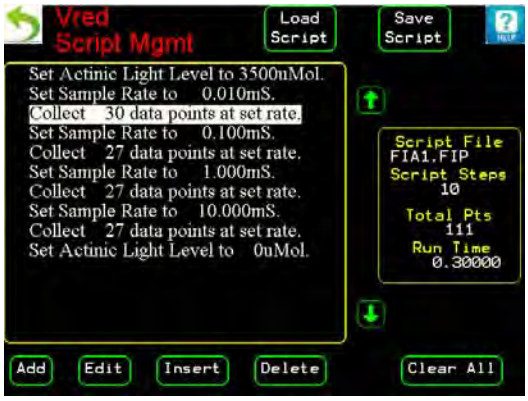
The **"Duration"** may be set from 1 second to 15 seconds.

The Presets or measuring parameters may be saved in a Preset file name for future retrieval by tapping **"Save Presets"**. Previous Preset files may also be selected and loaded into the instrument active memory by tapping **"Load Presets"**. If the Pre set file is only on the Data Card, the card must be put back into the instrument. When done with the set up parameters available from this screen, tap the return key in the upper left hand corner of the screen to get back to the main measuring window.

Tap the "Change Settings" button and tap **"Edit Script"**







Vred Script Mgmt

This **Vred Script Mgmt** screen allows the creation of custom measuring scripts.

The standard OJIP Script appears as a default script and it may be edited as desired. It is recommended that any edited scripts be saved with a new name to save the default script separately. To "Edit" the script, highlight the line of interest using the green up and down arrows and tap "Edit". A window that allows one to change values appears with an orange outline. Enter the desired value and tap the return key. To exit the orange window, tap the **"X"** in the upper right hand corner of the orange window.

To **"Add"** a function, tap "Add". A small screen will appear that will allow adding a **"Pt Rate"**, or sampling rate, **"Pt Count"** or the number of points to be captured in this section of the test, and **"Source"** for adding actinic light or far red light, and **"Cancel"**

"Insert" allows inserting a function above the highlighted line in the script window.

Here again, a small window will appear that will allow adding a "Pt Rate", or sampling rate, "Pt Count" or the number of points to be captured in this section of the test, and "Source" for adding actinic light or far red light, and "Cancel"

On the right are the Script file name, the number of steps in the script and the number of points measured, and the length of the test in time. Do not exceed 65,000 points.

There are safe guards that prevent illogical scripts. For example: a function must have a "Pt Rate", "Pt Count", and a source intensity even if that value is zero. In addition, there must be a script line somewhere before the end of the program that turns off the light sources. When finished tap "Save Script"

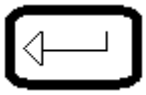
"Load Script" and "Save Script"

"Load Script" and "Save Script" The OS5p+ provides two types of files for this protocol, scrip files and data files. Measuring parameters, once set, are stored as script files by tapping "Save Script". To recall saved script files, tap "Load Script". Data files are created, recalled, and appended when the protocol is opened or under **Data Management "Log file Name"** If Measuring parameters are changed while using a specific data file, the new parameters are listed above the data in the data file, collected after the change, automatically. When saving a set of measuring parameters to a script file, tap the "Save Script" button and the options "New", "Existing" or "Cancel" will appear.

If **"New"** is selected, an alpha-numeric keyboard will appear and allow an 8 character name to be input. When finished, tap the return key to save the file. The suffix for Preset files is .FVM (for FV/FM, .YLD (for Y(II)), .KIN (for quenching), .OJP (for Strasser OJIP) .FIP (for the Vredenberg OJIP protocol). The suffix for data file names is .CSV .

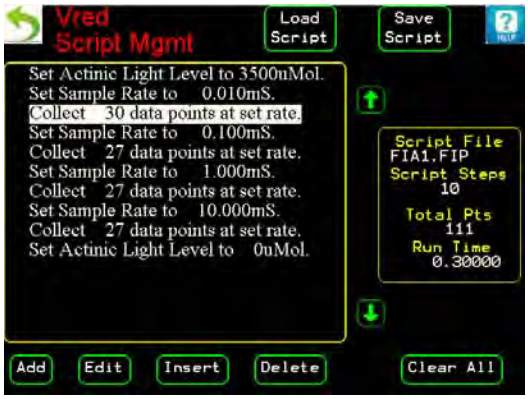
If **"Existing"** is selected, a previously made list of Preset files will appear. Select a file to be modified, or tap the "X" on the top right hand side of the orange box to cancel. If one has important Presets files or data files, copies should be saved to a data card for future retrieval. This way, if multiple researchers are using an instrument, the important measuring parameters and data can be saved safely.

When the parameters have been adjusted or changed for a specific set of conditions, the test setup can be saved by pressing "Save Preset" while in the Y(II) setup screen. A key board screen will appear, and the test set up can be named and it is saved when the return key is tapped on the key board. To exit the screen tap the return arrow in the upper left of the screen.



Return key

To load previously created tests, press "Load Script". Find the file of interest and tap the file of interest to load it. Tap the test of interest. After it is highlighted, tap the name of the file. The number of tests that can be saved is almost unlimited. Touch the top of the list to scroll up and the bottom of the list to scroll down. Exit if there is no action by tapping the "X" in the upper right hand corner of the orange box.



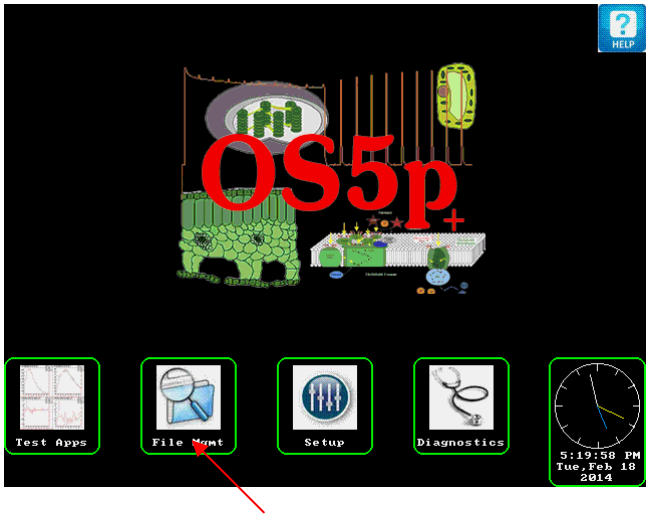
"Delete" allows the highlighted script line to be deleted. "Clear All" allows all script lines to be deleted.

Chapter 4 • OS5p+ Data Management Systems

Data Management

USB data transfer

Plug the USB cable into the OS5p+ and into your computer. Go to the Main Menu and press the **"File Mgmt"** button. A screen will appear that says not to disconnect the USB cable without ejecting the drive from you computer or tapping stop in place of eject..



A Screen will appear that says: **USB Remote PC Access Mode** V1.0The OS5p+ is now connected to your host PC. Ready to transfer data or update files. Use the PS eject feature to stop drive access. The Escape Key can be used if needed.

Do not disconnect the USB cable when data is being transferred or the file may be lost or corrupted.

File transfer by USB cable

Turn on the OS5p+ and press “File Mgmt”. Next, plug in the USB cable provided to a PC and the OS5p+. When the PC and the fluorometer are connected, the fluorometer screen will display a message titled:

USB Remote PC Access Mode V1.0 in a blue and a white colored message.

The OS5p+ is now connected to your PC.

You may transfer data or update files.

Use the drive eject feature to stop drive access.

Do not disconnect the unit without alerting the PC or data may be lost.

The stop key may be used if no Eject is found.

In Windows 8, or other Windows products, if a window does not appear on your PC screen go to Windows Explorer, then, under Computer, find the drive listed as

“OS5PII (Drive letter;)” left click this drive to open it with the mouse. Next left click the Excel data file of interest to open it with the mouse. The file may be saved on the PC as an Excel file. It has a comma delineated format.

Under these conditions, the fluorometer becomes a removable drive for the PC. The PC screen is now used to transfer files, not the OS5p+.

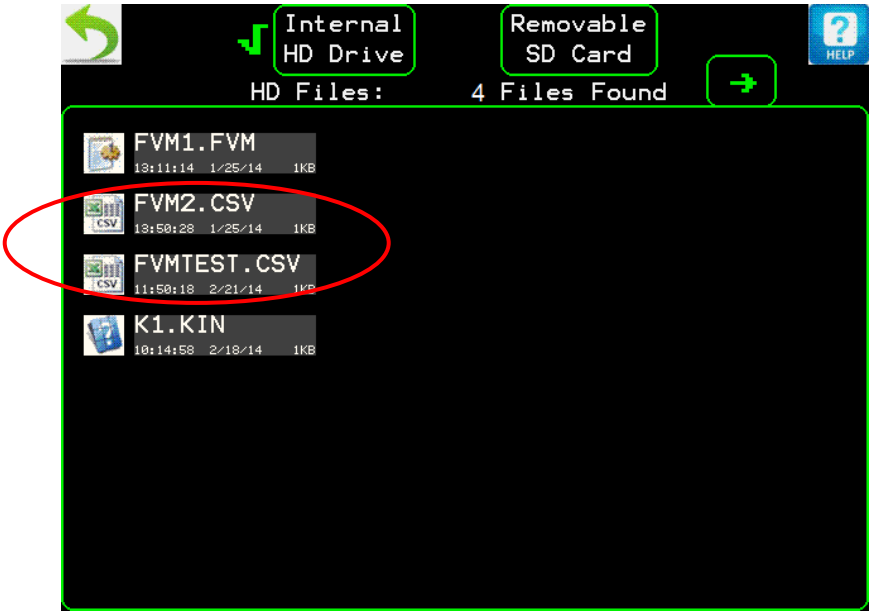
File Transfer by Data card

The OS5p+ comes with a 1 gigabyte data card; however, commercial 1 and 2 gigabyte data cards may be used with the OS5p+.

Insert the data card in the SD Card port.

Turn on the instrument

From the main instrument screen select **“File Mgmt”** and the following window will appear.



Files with the suffix **.CSV** on the OS5p+ are data files that are in an Excel format. The Suffix does not appear in the file name on a PC, only the Excel icon and the file name before the suffix. Files with a note pad and a gear that end in a suffix related to the measuring protocol are Preset files. Files that have a ? icon are of an unknown format. They may include pictures to be used as wall paper or other images. Files with suffixes like .FVM , .KIN , .YLD .RLC, .OJP and .FIP are Preset or script files. They may be opened on your computer using Microsoft Notepad.

To copy a file from the HD hard drive to a data card, tap the file of interest and a small window will appear asking to Delete, Copy or Cancel the file. Select Copy and the file will be copied to the data card. When finished. Push the data card into it's slot again and a spring will eject the data card from the instrument. Insert the card into a card reader on your PC (USB to PC card adapters are available for older PCs.) and go to Windows Explorer. Next go to Computer on your PC and find the Removable Disk that represents the Data Card. Open the file by left clicking the mouse. Files may the be selected. Data files are shown as Excel files.

To view files on the data card tap the **"Removable SD Card"** button.

Ejection process

When done using the SD card with your PC, go to the Removable Disk under Computer in Windows Explorer and right click the mouse over the Removable Disk. A menu will appear. Select Eject. When a message appears on your PC that it is safe to remove the data card, remove the data card.

Software Updates

From time to time software updates may be available for improvement of existing functionality or to increase instrument functionality.
To update software versions., contact Opti-Sciences Inc. 603-883-4400 www.optisci.com, or support@optisci.com , 8 Winn Ave. Hudson, NH 03051.

Appendix A • Maintenance

Your OS5p+ contains no user serviceable components. Please contact your closest authorized agent for periodic preventative maintenance and calibration information.

Note: opening the OS5p+ case voids the warranty!

This is a very reliable type of battery that exhibits no memory effects whatsoever. It is recommended that when the system is not in use, even for very long periods of time, the charger should be plugged in. Prolonged periods of discharge will significantly decrease its life span. Please contact Opti-Sciences if you have any questions concerning proper long term storage.

Note: DO NOT use an alternative battery charger.

All OSI systems are 19volt except the OS30p+.

Cleaning

The OS5p+ is made from durable materials, but some organic solvents can damage the surface finishes. Use a damp cloth with a mild detergent to clean the outside. The display window is made of a special, low reflectance touch screen, while the case is an ABS plastic. Should dirt get into the optical ports, (remember to use the protective caps supplied) clean the optics with a cotton swab and isopropyl alcohol. If the unit becomes submerged in water, return it to the factory for maintenance.

Miscellaneous Maintenance

Battery

The Sealed Nickel Metal Hydride Battery will provide about 8 to 10 hours of average use, depending primarily on use of the Saturation source lamp. Under extreme heat or extreme cold conditions, the battery charge life can be reduced. If field work is being done under these conditions, the 70 hour battery belt is recommended.

The typical recharging period is 10 hrs. Leaving the charger plugged in for longer than this will not damage the battery.

WARNING: Do not connect anything other than the provided charger to the charger jack, or you may damage the OS5p+!

As the battery ages, it may loose its ability to hold a charge. When this gets to about 1/2 its original life time or about 4 to 5 hours, send the unit to the factory for battery replacement. *Removing the battery and breaking the seal voids the warranty!!!*

Note: you can run the OS5p+ off the battery charger alone, however, it will not recharge while the instrument is on.

This is a very reliable type of battery that exhibits no memory effects whatsoever. It is recommended that when the system is not in use, it may be stored in the case. Prolonged periods of storage will discharge will discharge the battery. After the battery has fully charged, the charging cycle stops. The power cord must be unplugged and re-inserted to restart the charging cycle again. Long periods with out charging can significantly decrease its life span. Please contact Opti-Sciences if you have any questions concerning proper long term storage.

Light sources

All Light sources are LED rated for thousands of hours. Replacement must be done at the factory.

Trouble Shooting Power Problems

A majority of the problems encountered with the fluorometer are directly related to the battery, the circuit breaker, and the battery charger. The various sources (especially the saturation source) require a large amount of power to operate properly. Current models are supplied with a Nickel Metal Hydride Batteries. The charge life of the NMH battery is about 8 hours between changes under most conditions.

The charger should be plugged in when not in use. Prolonged periods of discharge will significantly decrease it’s life span.

Trouble Shooting Table

Do not use this instrument with chargers not supplied by Opti-Sciences Inc.

The OS5p+ is a 19volt system.

Symptom	Probable Cause	Cure
Unit will not turn on	• Dead battery	• charge battery or use AC power.
Unit resets when a test is run	• battery insufficiently charged • battery has become "weak" and needs replacement	• charge battery • Contact factory
Display dims or changes contrast during a test	• battery low	• charge battery • Contact Factory
Battery does not charge	• charger connector loose • charger is defective • dead battery	• check connections • replace charger • Contact factory

Remote trigger does not initiate a test	• remote trigger wire is not plugged in correctly • OS5p+ is not in proper test mode • fluorescence signal is too low	• check connections • Be sure that a cursor is not blinking in the test mode box. If it is, the test mode has not been entered. Press the test mode key to enter the test mode. • raise modulation intensity
Saturation light does not trigger	• fluorescence signal (Ft) Is too low. • if display also blanks, battery may be low	• increase the modulation intensity • Contact factory

Symptom	Probable Cause	Cure
Cannot output data to computer - or - Output to computer garbled	• cable is loose • hardware handshake protocol on host	• secure cable connections
Unit consistently loses data	• Flash memory defective	• contact nearest authorized agent for memory replacement
Fluorescence signal is noisy	• battery is insufficiently charged • external actinic light source is heavily modulated (pulsed xenon, or fluorescent light) • external illumination source has too much IR radiation • fiber not steady on sample	• charge battery • use actinic illuminator with a continuous output not modulated. Do not use fluorescent lights!!! • filter out excessive IR • use open body clip

Screen flickers.	• battery is insufficiently charged	• charge battery • Use battery charger to run the system.
Incorrect PAR reading with optional PAR cuvette attached	• PAR sensor not connected	• Secure the connection
Incorrect temperature reading with optional PAR cuvette attached	• Temperature sensor not connected	• Secure the connection

Appendix B• PAR Clip

PAR Clip

The PAR leaf Clip is designed to provide basic data to the OS5p+ system on photosynthetically active radiation and leaf temperature conditions. This clip resembles the standard 45^0 angle open body clip provided with the OS5p+, with two additional features, a thermistor for measuring leaf temperature and a PAR sensor for measuring light radiation on the leaf. The PAR sensor is mounted to a small support extending into the leaf measuring area. The thermistor is tethered below the support to measure leaf temperature. The thermistor is mounted in epoxy and it is non-destructive.

Unlike other brands of PAR Clips, the OS5p+ PAR Clip measuring jaw opens from the bottom. This prevents the unwanted opening of the PAR Clip when measuring high leaves or when the PAR Clip is used on a stand for longer quenching measurements.

The default equation for ETR when using the PAR Clip is:

ETR=Y(II) x PAR x 0.84 x 0.50. This equation uses average plant values for relative comparison between samples of the same species. For more exacting ETR values the leaf absorption and the ratio of PSII reaction centers may be changed in the various protocol to measured values. See the application note regarding PAR measurement for more details.

Connection

The fiber optic light guide is positioned in the clip in the same manner as the 45^0 clip. A nylon locking screw secures the fiber end. The PAR clip is connected to the "accessory" port on the OS5p+.

Technical specs

Weight:	0.6 Lbs. or .27kg
Cable length:	1 m.
Thread Mount for tripod	¼ inch 20 thread

Temperature Sensor: Thermister

Temperature Range	5~60 ^oC.
Accuracy	± 1.0 ^oC (worst case).
Resolution	± 0.1 ^oC.

PAR Sensor: GaAs sensor, Cosine Corrected

Range	0 ~ 3000 µE or µmls
Resolution	2 µE or µmls
Cosine Corrected	to 80 degrees

Both PAR Clips are calibrated To a Licor L-190 Quantum Sensor at the factory a standard traceable to National Institute of Standards and Technology. They should be returned to the factory every two years for recalibration.

Using the PAR Clip

The PAR clip must be connected to the accessory port on the OS5p+ when the unit is turned off.

The PAR Clip should continuously read different light values as PAR on the Y(II) and Quenching measuring screens as it moves from one light condition to another before a measurement. The PAR clip is required in quenching measurements. The PAR clip measures the internal white actinic light intensity incident on the sample used during the light adapting phase of a quenching protocol. It automatically adjusts the actinic light intensity to the programmed level and maintains that level to +-3 µmols over the entire light adapted phase, ensuring a stable actinic light output.

The PAR Clip is designed to measure actinic light intensity and record the average value just before the saturation pulse in the yield and tests. During the quenching test, it may register up to 2000 µmol during the saturation pulse, however, it is designed to record the actinic value just before the saturation pulse to the measuring file.

When moving from a dark clip to a PAR clip, the modulated light source will require adjustment in the test protocol that is being used. You may read the "fluorescence value too low" when the measuring button is pressed. This is because the modulated light source intensity needs to be adjusted upward. The OS5p+ has an automated modulated light adjustment capability in all measuring protocols. The PAR clip must be properly connected and a leaf, representative of the type to be measured, must be in the PAR clip leaf chuck as shown above. Then tap the Auto button in the protocol set up page. It may also be manually adjusted. Gain should be adjusted last. The lower the setting, the lower the signal to noise ratio. The modulation light source intensity should be set with a leaf in the leaf holder of the PAR clip. If it is too low then the "too low" message appears during measurement.

The setting can sometimes vary with different samples, for instance, leaves with a low chlorophyll content may require more modulated light intensity.

When the PAR Clip is used in ambient light it is important not to change distance or the orientation of the leaf in relation to the sun or external light source. The leaf has reached steady state at its current orientation and changing the angle of the leaf will cause plant mechanisms to start to adjust to the new actinic light level. Yield measurements are always done at steady state photosynthesis or errors will result. Reoriented leafs are not at steady state. According to Maxwell and Johnson it take between 15 and 20 minutes for most leaves to reach steady state; however, new data regarding chloroplast migration indicates that it may take from 20 minutes to 35 minutes to reach steady state at high light levels. (See the section on quantum yield of PSII for in depth discussion)

In addition, the PAR clip should be oriented on the leaf to minimize sample measuring area shading.

What is the value of a PAR clip in Photosynthesis measurement?

PAR clips have been used extensively for applications where ambient light and temperature can fluctuate. Since Y(II) measurements and other parameters, can change with light levels and temperature, the ability to study measurements as a function of PAR value or temperature can be valuable.

PAR clips have also been used for fixed level illumination studies and predetermined variable illumination studies as well. In these cases quenching studies, quenching relaxation studies, and plant performance at different light levels can be evaluated as a function of PAR and temperature. In order to measure ETR a PAR clip is necessary.

PAR Clips are also used for development of light curves. The PAR clip is an excellent choice for this type of work.

The Term PAR means photosynthetically active radiation in the wave band between 400-700 nm. PAR can be measured in different dimensions such as Watts per meter or in micro- Einsteins (µE) or micro-moles (µmls). When using a PAR Clip, dimensions will always be in the equivalent terms, micro-Einsteins, or micro-moles.

PPFD, or "photosynthetic photon flux density", is the number of PAR photons incident on a surface in time and area dimensions. These terms are equivalent for PAR Clip leaf radiation measurements. Furthermore, both can be presented in either of the equivalent dimensions, micro-moles or micro-Einsteins.

Both the PAR and PPFD terms have used extensively in biology. Micro-Einsteins, and micro-moles have also been used extensively in biology.

Appendix C • Technical Specifications

Measured and Calculated Parameters

F_O, F_O' F_M, F_V/F_M, F_M', F, Y(II) or ($\triangle F/F_M$') , PAR, leaf temperature.

Strasser protocol Parameters

t_{100}:s, t_{300}:s (or K step), t_{FM} (or time to P or FM), A (or area above the curve), M_O (or RC/ABS), & Pl_{ABS} (or performance index).
ABS/RC, TR_O/RC, DI_O/CS, ET_O/RC,TR_O/ABS, ET_O/TRO, ET_O/CS,RC/CS_O, RC/CS_M , S, M, T

Vredenberg protocol parameters

Ft/Fo

Eilers and Peters RLC measuring parameters

α = s or the slope of the initial curve rise

ETRMax = pm or the maximum electron transport rate, or optimal production

I_m = optimum illumination or PAR value at ETRMAX

I_k = characteristic intensity or the intensity where light saturation starts to dominate, or the minimum saturation level.

Kramer's equations

Y(II) = (F_M' -F_S)/F_M' or)F/ F_M'
qL = ((F_M'-F_S)/ (F_M'-F_O))(F_O'/F_S) or q_L = q_P(F_O'/F_S)
Y(NO) = 1/(NPQ+1+q_L (FM/F_O -1))
Y(NPQ) = 1- Y(II)-Y(NO)

Hendrickson protocol Parameters with NPQ resurrected from the puddle model by Klughammer

$Y(II) = (F_M' - F_S)/F_M'$ or $\Delta F/ F_M'$
$Y(NO) = F_S'/F_M$ or F'/F_M
$Y(NPQ) = (F_M/F_M') - Y(NO)$
$NPQ = Y(NPQ)/Y(NO)$ or $NPQ = (F_M-F_M')/F_M'$

Puddle model parameters

$q_P = (F_M'-F')/(F_M'-F_O)$ Above 0.4, Fo' should replace Fo
$q_N = 1- ((F_M'-F_O) / (F_M-F_O))$ Above 0.4, Fo' should replace Fo
$NPQ = (F_M-F_M')/F_M'$
$NPQ=q_E+q_M+q_I$ or $q_E+q_Z+q_I$ or $q_E+q_T+q_I$

Relaxation: test for Hendrickson with NPQ from Klughammer, and puddle model
$q_E = ((F_{ME}-F_{M'})/(F_M-F_{MS'}))$ is the relaxation saturation value
at four minutes to seven minutes in the dark. (Values can be changed).
$q_M = ((F_{MM}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at
twenty minutes in the dark. (Values can be changed).
$q_Z = ((F_{MZ}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at
twenty minutes in the dark. (Values can be changed).
$q_T = ((F_{MT}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at
twenty minutes in the dark. (Values can be changed).
$q_I = ((F_M-F_{MM}) / F_M-F_{M'}))$ Relaxation of qI starts at about forty minutes and can take up to sixty hours. qI can be determined from the dark adapted F_M measurement and the saturation pulse at q_M , or q_Z , or q_T. (Values and times can be changed).

PAR, Leaf temperature, estimated ETR (using Standard PAR Clip).

Light Sources

Saturation pulse: White light LED with 690 nm short pass filter. Adjustable 0 ~ 15,000 µmol, or 0-7,500 µmol with a PAR Clip

Modulated light: 660 nm LED with 690 nm short pass filter. Adjustable 0 ~ 1 µmol

Modulated light: 450 nm LED. Adjustable 0 ~ 1 µmol

Actinic illumination: White light LED. Adjustable 0 ~ 1,850 µmols with PAR Clip

Actinic illumination: For OJIP, White light LED 0 ~ 5,800 µmols

Optional Red LED Actinic light for OJIP only. It must be ordered at time of purchase (replaces white LED) 0 ~ 6,000 µmols

Far red: 735 nm LED with red cut off filter (Used for F_O' determination In Kramer lake model, and puddle model parameters, and for re-oxidizing PSII before F_V/F_M measurements). Intensity and duration adjustable. In the quenching protocols it may be turned off or on for any phase of a test, multiple phased or it may be on or off for all phases of a test. Intensity adjustment from 0-15 mW/m^2 .

Far red light is removed from the white LED actinic light to provide greater experimental control. It may be added as necessary in the various measuring protocols to excite photosystem I, or turned off to not excite photosystem I.

Detector and Filters

A PIN photodiode with a 700 ~ 750 nm band pass filter.

Modulation frequency

Selectable from 25 Hz to 1 MHz with auto switching based on phase of test for both red and blue light sources.

Filter constants

Automated from 1µs to 1 S with auto switching based on phase of test.

Sampling Rate

Variable from 1 to 1,000,000 points per second with auto switching based on phase of test.

Test Duration

Adjustable from 2 seconds ~ 10 hours (Longer with adapter and AC source).

Processor

ARM 7

Storage Capacity

1 GByte internal flash memory almost unlimited testing.

Data cards of 1 and 2 GByte data card size can be used for data storage as well.

Digital Output

USB, MMC/SD data card with up to 1 GByte data card size,

User Interface

Color graphic touch screen.

Power Supply

Internal rechargeable sealed Nickel Metal Hydride battery.

Battery Life

8 hours of continuous operation.

Dimensions

7.75 in x 6 in x 4.5 in. in carrying bag

Weight

2.72 kg or 6.0 lbs with Fiber optic and PAR Clip included

Once the battery charge cycle is complete, the charger stops charging. The power cord must be unplugged and then plugged in again to start recharging. Over time, the battery will lose charge if it remains plugged in or not. Do not let the instrument sit without recharging for extended periods of time. This will shorten battery life. Breaking the seal on the chlorophyll fluorometer will void the warranty. Batteries should be replaced at the factory or by an OSI distributor after the end of the warranty period.

Battery charge life will be shorted by extreme heat or cold. Consider purchasing the 70 hour battery belt if the instrument is to be used under these conditions.

Appendix D • Data Formats

Overview

Each test has a unique data output format, tailored to the changing measured parameters. The differences between the data groups are listed in this section. Measuring data may be transferred in a spread sheet format and traces are presented in a vertical sequential list of intensity values that may be graphed in Excel or other spread sheet products.

Data exists in two forms in the OS5p+. The first type is the fluorescence signal over the course of a test. This "raw data" is useful when visualizing what is happening dynamically in the photo-system, as it is exposed to the different illumination sources over the course of the test. The second kind of data is the tabular data that is generated around the saturating pulses for test modes. The specifics of each of these parameters are mentioned later in this section. Storage method varies between these two types of data. The “raw data” for a trace is allotted a unique storage number based on the next open location in the storage directory, as they are saved. The user can not specify which slot number a trace is stored to. The "Tabulated Data", on the other hand, is stored in logical groupings called file numbers. Unlike traces, the current file number is a user set-able option. When joint saving of trace and data is desired, the file number can be chosen to match the “Next Trace Slot” shown in the “Menu trace save function”. This will give the file and trace the same identifying number. There can be almost an unlimited number of different files and traces stored simultaneously in the system depending on memory use. Information on free memory status is always available in the “File menu” management utilities screen of each test mode. Traces and tabulated data are always saved together in quenching tests, There are specific formats for each type of data and trace. Each test mode will generate a slightly different data output (each test measures different parameters). The details of each type are listed at the end of this section.

All types include a common header containing information about the date, time, machine number, and setup of the machine when each measurement was made.

Data Format information:

Data formats for test protocols are listed below. All data types and output methods are presented in a ASCII format that is comma delimited with a character 13 (<CR.>) code at the end of each line. This type of data output format, while bulkier than binary coding, is compatible with most data analysis and spreadsheet programs "Import" feature.

F_V/F_M data file format

!FVFM 10SEC FARRED TEST			←	Note											
5p+ Fv/m Test															
Sample #	Date	Time	Mod Int	Mod WI	Det Gain	Sat Flash	Sat PW	Far Red Mode	Far Red Int	Far Red Dur	Fo	Fm	Fv	Fv/m	Fv/o
1	7-Mar	15:16	80	R	11	100	0.8	On	100	10	727	2266	1539	0.679	2.116
2	7-Mar	15:20	80	R	11	100	0.8	On	100	10	731	2263	1532	0.676	2.095
3	7-Mar	15:26	80	R	11	100	0.8	On	100	10	734	2269	1535	0.676	2.091
!FIELD12			←	Note											
4	7-Mar	15:31	80	R	11	100	0.8	On	100	10	735	2267	1532	0.675	2.084
!FIELD12 SAMPLE2			←	Note											
5	7-Mar	15:32	80	R	11	100	0.8	On	100	10	723	2257	1534	0.679	2.121
6	7-Mar	15:33	80	R	11	100	0.8	On	100	10	711	2244	1533	0.683	2.156

Y(II) data file format

!LAB3 SAMPLE 1 ← Note																					
!TREATMENT 7C ← Note																					
5p+ Y(II) Test																					
Sample #	Date	Time	Mod Int	Mod WI	Det Gain	Sat Flash	Sat Width	Flash Mod	Far Red M	Far Red In	Far Red Di	Pre-Illum N	Illum Dur	Ke	Kq	PAR Corr	F'	Fm'	Y(II)	ETR	PAR	Temp
1	7-Mar	15:59	80	R	8	100	0.8	S	Off	50	5	Off	1	0.5	0.84	1	575	1536	0.625	2.3	9	21.5
!SAMPLE 21 ← Note																					
!TREATMENT 7D ← Note																					
2	7-Mar	16:00	80	R	8	100	0.8	S	Off	50	5	Off	1	0.5	0.84	1	580	1530	0.62	2.3	9	21.5
!SAMPLE 5 TREATMENT 7C ← Note																					
3	7-Mar	16:02	80	R	8	100	0.8	S	Off	50	5	Off	1	0.5	0.84	1	578	1525	0.62	2.3	9	21.5
!SAMPLE 2 ← Note																					
!TREATMENT 7B ← Note																					
4	7-Mar	16:03	80	R	8	100	0.8	S	Off	50	5	Off	1	0.5	0.84	1	582	1521	0.617	2.3	9	21.5

- **Multiple notes may be left for every measurement, or one 32 characher note.**

Ke = Ratio of PSII reaction centers to PSI reaction centers

Kq = Leaf absorptance

Flash Mode is S for square topped or MulLvl for Multiflash

Far red Mode is on, off, or pre.

Quenching data file format – Hendrickson – with Klughammer NPQ & quenching relaxation

!HENDRICKSON REL QM 36 ← NOTE																		
5p+ Quenching Test																		
Mod Int	Mod WI	Det Gain	Sat Flash	Sat Width	Flash Mod	Far Red Int	Far Red Dur	Far Red Mode	Ke	Kq	PAR Corr	Calc Mode	Relax Phase	qE Time	q. Time	Relax Probe	Probe Time	Probe Count
80	R	4	100	0.8	S	50	5	Pre&All	0.5	0.84	1	Hendrk	On	4	36	On	4	9
Fo	Fm ← F_M & F_O																	
283	1150																	
Sample #	Date	Time	PAR	Leaf Temp	F'	Fm'	Fo'	Y(II)	ETR	Y(NO)	Y(NPQ)	NPQ						
1	7-Mar	12:59	398	21.2	587	604	0	0.028	4.6	0.51	0.461	0.9						
2	7-Mar	13:01	397	21.2	565	583	0	0.03	5	0.491	0.478	0.97						
3	7-Mar	13:03	397	21.2	554	572	0	0.031	5.1	0.481	0.487	1.01						
4	7-Mar	13:05	397	21.3	546	563	0	0.03	5	0.474	0.495	1.04						
5	7-Mar	13:07	397	21.3	537	557	0	0.035	5.8	0.466	0.498	1.06						
6	7-Mar	13:09	397	21.3	532	551	0	0.034	5.6	0.462	0.503	1.08						
7	7-Mar	13:11	397	21.3	525	544	0	0.034	5.6	0.456	0.509	1.11						
8	7-Mar	13:13	397	21.3	522	539	0	0.031	5.1	0.453	0.515	1.13						
9	7-Mar	13:15	397	21.4	516	534	0	0.033	5.4	0.448	0.518	1.15						
10	7-Mar	13:17	398	21.4	508	529	0	0.039	6.4	0.441	0.519	1.17						
11	7-Mar	13:19	398	21.4	506	524	0	0.034	5.6	0.44	0.525	1.19						
12	7-Mar	13:21	398	21.4	501	519	0	0.034	5.6	0.435	0.53	1.21						
13	7-Mar	13:23	398	21.4	494	515	0	0.04	6.6	0.429	0.53	1.23						
14	7-Mar	13:25	398	21.4	491	510	0	0.037	6.1	0.426	0.536	1.25						
15	7-Mar	13:27	398	21.5	487	506	0	0.037	6.1	0.423	0.539	1.27						
16	7-Mar	13:29	398	21.5	481	501	0	0.039	6.4	0.418	0.542	1.29						
17	7-Mar	13:31	398	21.5	476	498	0	0.044	7.3	0.413	0.542	1.3						
18	7-Mar	13:33	397	21.5	471	494	0	0.046	7.6	0.409	0.544	1.32						
19	7-Mar	13:37	0	0	268	667	0	0	0	0	0	0						
20	7-Mar	13:41	0	0	276	754	0	0	0	0	0	0						
21	7-Mar	13:45	0	0	277	795	0	0	0	0	0	0						
22	7-Mar	13:49	0	0	275	813	0	0	0	0	0	0						
23	7-Mar	13:53	0	0	275	826	0	0	0	0	0	0						
24	7-Mar	13:57	0	0	276	836	0	0	0	0	0	0						
25	7-Mar	14:01	0	0	280	844	0	0	0	0	0	0						
26	7-Mar	14:05	0	0	278	848	0	0	0	0	0	0						
27	7-Mar	14:09	0	0	277	854	0	0	0	0	0	0						
FmsS	FmsE	FmsM	qE	qM	ql													
494	667	854	.35	.37	.59													
Curve:																		
1150																		
1150																		
1043																		
798																		
697																		
655																		
634																		
617																		
607																		
601																		
597																		
594																		
604																		

Sample # refers to saturation flash #

Time between saturation flashes is indicated by time.

Fluorescent curve raw data continues for the entire measurement protocol. It can be graphed using Excel graph wizard.

Page 1

Quenching data file format – Kramer with quenching relaxation

5p+ Quenching Test																		
Mod Int	Mod WI	Det Gain	Sat Flash	Sat Width	Flash Mode	Far Red Int	Far Red Dur	Far Red Mode	Ke	Kq	PAR Corr	Calc Mode	Relax Phase	qE Time	q. Time	Relax Prot	Probe Time	Probe Count
80	R	8	100	0.8	S	50	5	Off	0.5	0.84	1	Kramer	On	4	36	On	4	9
Fo	Fm																	
263	1103																	
Sample #	Date	Time	PAR	Leaf Temp	F'	Fm'	Fo'	Y(II)	ETR	Y(NO)	Y(NPQ)	NPQ	qL					
1	3/17	15:50	300	21.9	531	578	239	0.081	10.2	0.498	0.421	0.9	0.034					
2	3/17	15:54	301	21.9	525	570	241	0.078	9.8	0.508	0.414	0.93	0.011					
3	3/17	15:58	301	21.9	524	559	250	0.062	7.8	0.405	0.533	0.97	0.156					
4	3/17	16:02	302	22	515	561	245	0.081	10.2	0.418	0.501	0.96	0.135					
5	3/17	16:06	302	22	508	550	245	0.076	9.6	0.374	0.55	1	0.21					
6	3/17	16:10	301	22	508	549	240	0.074	9.3	0.344	0.582	1	0.284					
7	3/17	16:14	301	22	499	545	241	0.084	10.5	0.315	0.601	1.02	0.361					
8	3/17	16:18	301	22	494	541	235	0.086	10.8	0.377	0.537	1.03	0.195					
9	3/17	16:22	301	22	493	535	241	0.078	9.8	0.399	0.523	1.06	0.139					
10	3/17	16:26	0	0	260	790	0	0	0	0	0	0	0					
11	3/17	16:30	0	0	263	850	0	0	0	0	0	0	0					
12	3/17	16:34	0	0	257	844	0	0	0	0	0	0	0					
13	3/17	16:38	0	0	258	857	0	0	0	0	0	0	0					
14	3/17	16:42	0	0	253	863	0	0	0	0	0	0	0					
15	3/17	16:46	0	0	252	874	0	0	0	0	0	0	0					
16	3/17	16:50	0	0	256	880	0	0	0	0	0	0	0					
17	3/17	16:54	0	0	257	894	0	0	0	0	0	0	0					
18	3/17	16:58	0	0	256	917	0	0	0	0	0	0	0					
FmsS	FmsE	FmsM	qE	qM	ql													
535	790	917	0.47	0.23	0.34													
Curve:																		
	1103																	
	1103																	
	855																	
	637																	
	583																	
	556																	
	548																	
	547																	
	546																	
	552																	
	548																	
	540																	
	536																	
	541																	
	538																	

Raw flurorescence curve data may be graphed in Excel. All of the data is available. This picture shows an abbreviated amount of data.

Kramer lake model equation data file.

Times are for saturation pulses.

NPQ is reported but it is part of the Hendrickson - Klughammer lake model equations.

Quenching relaxation values relates to NPQ

qM was sellected for this test

Quenching data file format – Puddle model with quenching relaxation

5p+ Quenching Test																		
Mod Int	Mod WI	Det Gain	Sat Flash	Sat Width	Flash Mode	Far Red Int	Far Red Dur	Far Red Mode	Ke	Kq	PAR Corr	Calc Mode	Relax Phase	qE Time	q. Time	Relax Probe	Probe Time	Probe Count
80	R	7	100	0.8	S	50	10	On_All	0.5	0.84	1	Puddle	On	4	36	On	4	8
Fo	Fm																	
135	547																	
Sample #	Date	Time	PAR	Leaf Temp	F'	Fm'	Fo'	Y(II)	ETR	qP	qN	NPQ						
1	4/15	14:04	132	22.2	255	307	119	0.169	9.3	0.302	0.561	0.78						
2	4/15	14:08	132	22.2	246	303	117	0.188	10.4	0.339	0.568	0.8						
3	4/15	14:12	132	22.3	243	306	119	0.205	11.3	0.368	0.564	0.78						
4	4/15	14:16	131	22.3	235	305	121	0.229	12.5	0.38	0.569	0.79						
5	4/15	14:20	131	22.3	236	306	121	0.228	12.5	0.378	0.566	0.78						
6	4/15	14:24	131	22.3	230	304	122	0.243	13.3	0.406	0.572	0.79						
7	4/15	14:28	131	22.3	228	301	120	0.242	13.3	0.403	0.577	0.81						
8	4/15	14:32	131	22.4	224	299	118	0.25	13.7	0.414	0.579	0.82						
9	4/15	14:36	0	0	131	440	0	0	0	0	0	0						
10	4/15	14:40	0	0	133	465	0	0	0	0	0	0						
11	4/15	14:44	0	0	135	478	0	0	0	0	0	0						
12	4/15	14:48	0	0	132	487	0	0	0	0	0	0						
13	4/15	14:52	0	0	134	494	0	0	0	0	0	0						
14	4/15	14:56	0	0	133	504	0	0	0	0	0	0						
15	4/15	15:00	0	0	133	506	0	0	0	0	0	0						
16	4/15	15:04	0	0	133	515	0	0	0	0	0	0						
FmsS	FmsE	FmsM	qE	qM	ql													
299	440	515	0.47	0.25	0.1													
Curve:																		
	135																	
	135																	
	547																	
	547																	
	458																	
	387																	
	338																	
	320	← Fluorescence trace data																
	304																	
	293																	
	287																	
	282																	
	279																	
	273																	
	272																	
	269																	
	266																	
	266																	
	263																	
	264																	

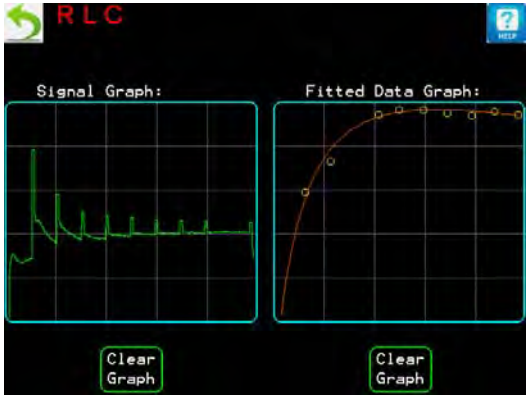
RLC – Rapid Light Curve Protocol data file format

5p+ RLC Test											
Mod Int	Mod WI	Det Gain	Sat Flash	Sat Width	Flash Mod	Far Red M	Far Red In	Far Red Di	Ke	Kq	PAR Corr
80	R	10	100	0.8	S	Off	50	5	0.5	0.84	1
Sample #	Date	Time	Dwell	PAR	Leaf Temp	F'	Fm'	Y(II)	ETR		
1	4/14	15:21	10	19	22.4	258	765	0.662	5.2		
2	4/14	15:22	10	49	22.4	446	617	0.277	5.6		
3	4/14	15:22	10	68	22.4	414	543	0.237	6.7		
4	4/14	15:22	10	99	22.4	422	516	0.182	7.5		
5	4/14	15:22	10	149	22.4	440	501	0.121	7.5		
6	4/14	15:22	10	199	22.5	446	493	0.095	7.9		
7	4/14	15:23	10	299	22.5	459	487	0.057	7.1		
alpha	Ik	ETRmax	Im								
0.609	12.3	7.5	230.3								
5p+ RLC Test											
Mod Int	Mod WI	Det Gain	Sat Flash	Sat Width	Flash Mod	Far Red M	Far Red In	Far Red Di	Ke	Kq	PAR Corr
80	R	10	100	0.8	S	Off	50	5	0.5	0.84	1
Sample #	Date	Time	Dwell	PAR	Leaf Temp	F'	Fm'	Y(II)	ETR		
1	4/14	15:23	10	18	22.4	218	610	0.642	4.8		
2	4/14	15:24	10	49	22.4	351	582	0.396	8.1		
3	4/14	15:24	10	69	22.4	366	523	0.3	8.6		
4	4/14	15:24	10	99	22.5	389	497	0.217	9		
5	4/14	15:24	10	149	22.5	412	487	0.154	9.6		
6	4/14	15:24	10	200	22.5	429	480	0.106	8.9		
7	4/14	15:25	10	300	22.5	443	477	0.071	8.9		
alpha	Ik	ETRmax	Im								
0.446	21.1	9.4	146.2								

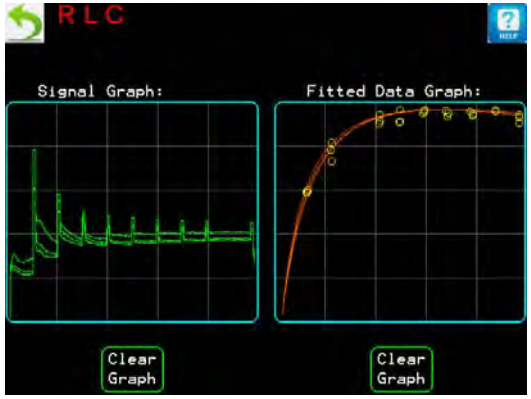
To save and view the Eilers and Peeters curve fitting graph, tap the help button in the upper right hand corner of the graph page after a RLC has been measured. Help will appear, and allow the image of the fluorescence trace and the fitted curve to be stored as a bit map file. The file may be opened as a jpeg, or a bitmap on your computer for additional work.

See the next page.

RLC graphs shown below are examples of images that may be captured and stored on the OS5p+:



A single graph



Multiple graphs overlaid

Strasser OJIP Protocol data file format

Sample #	Date	Time	Mod Int	Mod WI	Det Gain	Actinic Lvl	Run Time	O	t100	K	J	I	P	S	M	T	tPm	Area	PI	Vj	Mo	ABS/RC	TRo/RC	DIo/CS	ETo/RC	TRo/ABS	ETo/TRo	ETo/CS	RC/CSo	RC/CSm
1	7-Mar	17:14	40	R	1	3500	3	233	232	269	439	765	959	958	946	0	928	28548	10.469	0.284	0.161	0.751	0.1156	0.1825	0.4073	0.757	0.7163	126.34	310.224	1276.845
2	7-Mar	17:21	40	R	1	3500	3	250	258	310	551	842	967	963	953	0	262	13678	4.203	0.42	0.293	0.942	0.1702	0.2437	0.4055	0.741	0.5802	107.549	265.255	1026.005
3	7-Mar	17:28	40	R	1	3500	3	232	237	274	443	765	954	950	954	0	1150	29168	8.279	0.292	0.201	0.909	0.1423	0.2211	0.4871	0.756	0.7078	124.268	255.137	1049.139
Curve:	Time	Signal																												
	0.00001	229	246	228																										
	0.00002	233	250	232																										
	0.00003	238	255	237																										
	0.00004	241	258	239																										
	0.00005	240	258	238																										
	0.00006	237	257	237																										
	0.00007	235	256	237																										
	0.00008	233	256	236																										
	0.00009	232	257	236																										
	0.0001	232	258	237																										
	0.00011	233	259	238																										
	0.00012	234	262	240																										
	0.00013	235	265	241																										
	0.00014	236	266	242																										
	0.00015	239	266	243																										
	0.00016	241	267	244																										
	0.00017	243	269	247																										
	0.00018	246	271	252																										
	0.00019	248	274	255																										
	0.0002	249	278	255																										
	0.00021	250	282	254																										
	0.00022	252	286	255																										
	0.00023	254	289	257																										
	0.00024	256	291	258																										
	0.00025	258	293	259																										
	0.00026	260	293	261																										
	0.00027	263	295	265																										
	0.00028	265	298	268																										
	0.00029	267	307	271																										
	0.0003	269	310	274																										
	0.0004	289	343	294																										
	0.0005	303	365	304																										
	0.0006	317	386	318																										
	0.0007	330	404	333																										
	0.0008	343	420	346																										
	0.0009	353	435	358																										
	0.001	362	451	368																										
	0.0011	371	466	376																										
	0.0012	380	481	386																										
	0.0013	390	494	396																										
	0.0014	399	504	404																										
	0.0015	406	513	411																										
	0.0016	413	524	417																										
	0.0017	417	534	426																										
	0.0018	422	541	434																										
	0.0019	430	546	438																										
	0.002	439	551	443																										
	0.0021	445	557	447																										

Sample measuring time

0.00001 = 10 µsecs

Test 1

Test 2

Test 3

$PI = PI_{ABS}$

This is an example of an OJIP data file with the Log mode in the set up file set to grouped. If it is set to standard only one measurement trace can be recorded at a time and they will not be recorded in consecutive columns as they are in grouped.

Grouped mode allows multiple overlay graphing.

Both standard and grouped mode alow star charts also known as "radar charts" of different measurements parameters in Excel.

Use X Y Scatter graphs in Excel for OJIP overlay graphing and
Use Radar Charts in Excel for Star charts of measuring parameters shown above.

Test 1

Test 2

Test 3

Vredenberg OJIP Quenching Protocol data file format

!VINE SAMPLE DROUGHT STRESS 7 DAYS											
5p+ Vred OJIP											
Date	Time	Script	Mod Int	Mod Wl	Det Gain	Filtering	Far Red Mode	Far Red Int	Far Red Dur	Fo
3/17	13:20	JSTEPTST.FIP	40	R	3	None	Off	50	10	289
Curve:	Time(mS)	Ft	Ratio Ft/Fo							
	0.01	289	1							
	0.02	299	1.034							
	0.03	305	1.055							
	0.04	308	1.065							
	0.05	310	1.072							
	0.06	312	1.079							
	0.07	315	1.089							
	0.08	319	1.103							
	0.09	322	1.114							
	0.1	325	1.124							
	0.11	327	1.131							
	0.12	329	1.138							
	0.13	330	1.141							
	0.14	333	1.152							
	0.15	342	1.183							
	0.16	351	1.214							
	0.17	357	1.235							
	0.18	362	1.252							
	0.19	366	1.266							
	0.2	367	1.269							
	0.21	366	1.266							
	0.22	371	1.283							
	0.23	383	1.325							
	0.24	391	1.352							
	0.25	395	1.366							
	0.26	402	1.391							
	0.27	406	1.404							
	0.28	409	1.415							

One or more notes can be typed into the data file for each measurement

The script name is listed as JSTEPST.FIP . A description of the script can be viewed on the instrument by going to "File Mgmt", tapping on the script file name with an .FIP suffix and tapping "View".

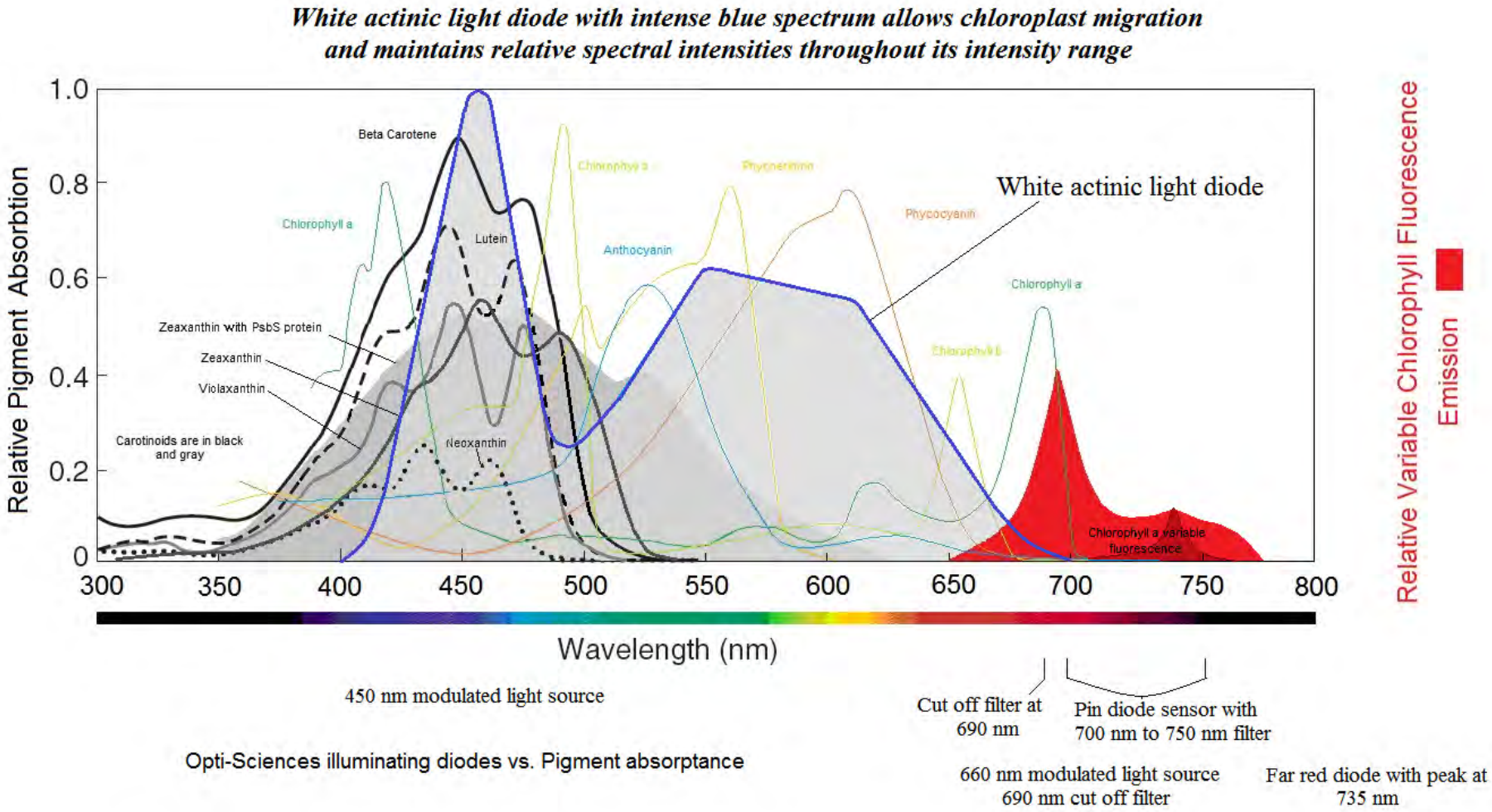
```
I3000
R00000010
S00200
I0000
R00000100
S00027
R00001000
S00027
R00010000
S00027
R00100000
S00027
End
```

Vredenberg OJIP Script found in .FIP suffix files.

It may be opened using Microsoft Wordpad on your computer.

I = Intensity
R = Rate
S = number of Steps or points
F = Far red light intensity

OS5p+ Spectral chart



Relative absorbtion of Zeaxanthin, and Zeazanthin bound to PSbS protien are adapted from Aspinall-O'dea M.(2002) Chlorophyll a, & b absorbtion spectra, chlorophyll a emission spectra, anthocyanin absorbtion spectra are adapted from Papageorgiou & Govinjee (2004). Relative absorption of Lutein, Beta Carotene, Neoxanthin, and Violaxanthin adapted from Lichtenthaler 2001